University of Alberta

BIOLISTIC TRANSFECTION OF IMMUNOMODULATING GENES INTO MOUSE ISLETS TO PROMOTE ALLOGRAFT SURVIVAL

by

ANITA LILLIAN GAINER ©

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A L Gainer

8364-120 St
Edmonton, Alberta T6G 1X2

April 18, 1997

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **BIOLISTIC TRANSFECTION OF IMMUNOMODULATING GENES INTO MOUSE ISLETS TO PROMOTE ALLOGRAFT SURVIVAL** submitted by **ANITA LILLIAN GAINER** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in **EXPERIMENTAL SURGERY.**

Dr. G.L. Warnock

Dr. J.E. Elliott

Dr. R.V. Rajotte

Dr. K. Kane

Dr. A. Rabinovitch

Dr. R.G. Gill

March 24, 1997

This text is dedicated to

Peter, Andrew and Ian

who endured the loss of the wife and mother they once knew

and to

Margaret Irving and Lara Hale

who helped maintain my perspective on the truly important things in this life

ABSTRACT

Localized delivery of immunosuppressive molecules, limited to the graft site, may allow transplantation of tissue in the absence of systemic immunosuppressive agents. To investigate this approach we required an efficient method to transfect intact islets. The biolistic method transforms cells by bombarding them with microprojectiles coated with DNA. Once internalized, the DNA is solubilized and expressed.

We compared transfection efficiencies of the biolistic method, lipofection and recombinant adenoviral infection. The biolistic method achieved a 35-fold higher level of reporter gene activity than the lipofection method; adenoviral infection achieved a further 25-fold increase. Control and biolistically-transformed islets responded similarly to an in vitro glucose challenge. Syngeneic, biolistically-transfected islets functioned in vivo to reverse the diabetic state.

We then tested the survival of purified CBA (H-2^k) islets, biolistically transfected with a cDNA encoding human CTLA4-Ig under control of the human CMV immediate early promoter, in allogeneic alloxan-induced diabetic BALB/c (H-2^d) recipients receiving no systemic immunosuppression. Functional survival was demonstrated in 50% of grafts transfected with CTLA4-Ig until follow up was concluded at 50, 130 or 165 days. Immunohistochemistry showed well-granulated, insulin-positive islets lying adjacent to, but not infiltrated by, dense aggregates of mononuclear cells.

We subsequently increased our biolistic transfection efficiency through improvement of our biolistic blasting conditions. The optimal helium pressure, gold particle size, number of islets used per blast and the optimal sample loading procedure were determined.

With the improved methodology, islet allograft survival within the same mouse model was determined with islets biolistically transfected with human CTLA4-Ig, human soluble Fas ligand, or a combination of the two molecules. Graft survival beyond 45 days

was 50%, 80% and 43% in the CTLA4-Ig, soluble Fas ligand and CTLA4-Ig/Fas ligand groups, respectively. Measurement of expressed protein by ELISA revealed very variable transfection efficiencies within all groups.

We conclude that the local production of human CTLA4-Ig and/or human soluble Fas ligand by biolistically-transfected islets promotes allograft survival. The success rate is at least partially limited by the transfection efficiency, which is determined by technical limitations of our current biolistic technology. Improvements in biolistic technology may allow greater success rates in the future.

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TABLE OF CONTENTS

I. GENERAL INTRODUCTION PAGE

DIABETES MELLITUS 1

ISLET TRANSPLANTATION 3
  1. Advantages 3
  2. Past experience 4
  3. Outstanding problems 5

CELLULAR IMMUNOMODULATION TO OVERCOME ISLET ALLOGRAFT REJECTION 6

THEORY OF GENE THERAPY (MOLECULAR IMMUNOMODULATION) IN ISLET TRANSPLANTATION 7

GENERAL TRANSFECTION METHODS 8
  1. Non-viral methods of gene transfer 9
  2. Viral methods of gene transfer 12

TRANSFECTION EXPERIENCE RELATED TO ISLET TRANSPLANTATION 15

CANDIDATE MOLECULES FOR IMMUNE INTERFERENCE BY GENE THERAPY IN ALLOGRAFT REJECTION 18
  1. The prevention of T_H cell activation 18
    B7:CD28 interactions 19
    CTLA4 20
    B7:CTLA4-Ig intereactions 20
    Other potential interactions for targeting 22
  2. Removal or neutralization of effector lymphocytes 23
    Fas receptor 24
    Fas ligand 25
    FasR:FasL interactions 26

Neutralization of effector cytokines.............................. 28
3. Reprogramming of the overall immune response through production of immune-modulating cytokines....................... 28

PURPOSES AND GENERAL OUTLINE OF THESE STUDIES........... 29

REFERENCES.. 35

II. SUCCESSFUL BIOLISTIC TRANSFORMATION OF MOUSE PANCREATIC ISLETS WHILE PRESERVING CELLULAR FUNCTION

INTRODUCTION.. 50

MATERIALS AND METHODS.. 51
1. Islet isolation... 51
2. Molecular constructs.. 51
3. Transfection methods.. 51
4. Electron microscopy studies.. 53
5. Characterization of biolistically transfected islets................... 53
6. Luciferase assays... 54
7. Functional studies.. 55

RESULTS.. 55
1. Optimization of the biolistic procedure.............................. 55
2. Particle penetration.. 56
3. Recovery and efficacy of transfection................................ 56
4. Functional studies.. 57

DISCUSSION.. 57

REFERENCES.. 67

III. EXPRESSION OF CTLA4-Ig BY BIOLISTICALLY TRANSFECTED MOUSE ISLETS PROMOTES ISLET ALLOGRAFT SURVIVAL

INTRODUCTION.. 69

MATERIALS AND METHODS.. 70
    1. Islet isolation.. 70
    2. Molecular constructs.. 70
    3. Transfection methods.. 71
    4. Functional studies... 71
    5. Histologic methods.. 71

RESULTS... 72
    1. Allograft survival.. 72
    2. Histological appearance of grafts.................................. 73

DISCUSSION.. 74

REFERENCES.. 80

IV. OPTIMIZATION OF THE BIOLISTIC TRANSFECTION PROCESS IN MOUSE ISLETS

INTRODUCTION.. 82
    1. Principle.. 83
    2. Optimization... 85

MATERIALS AND METHODS.. 86
    1. Islet isolation.. 86
    2. Molecular constructs.. 87
    3. Biolistic transfection.. 87
    4. Reproducibility of blasting procedure............................. 88
    5. Viability staining... 89
    6. Luciferase assays.. 89

RESULTS 90
1. Effect of the number of islets exposed per biolistic blast 90
2. Effect of different helium pressures per blast 90
3. Effect of gold particle size 91
4. Effect of sample loading procedure 91

DISCUSSION 93

REFERENCES 106

V. PROLONGATION OF ALLOGRAFT SURVIVAL OF BIOLISTICALLY TRANSFECTED ISLETS EXPRESSING HUMAN CTLA4-Ig, HUMAN SOLUBLE FAS LIGAND OR A COMBINATION OF THE TWO.

INTRODUCTION 108

MATERIALS AND METHODS 109
1. Islet isolation 109
2. Molecular constructs 110
3. Biolistic transfection 111
4. Islet transplants 112
5. Insulin determinations 112
6. ELISAs 113
i) Human CTLA4-Ig 113
ii) Human Fas ligand 114
7. Histologic methods 114

RESULTS 115
Optimizing the amount of DNA and gold particles used for transfection 115
1. Assessing islet damage by insulin levels 115
2. Levels of transfected gene expression 116
Transfection control grafts 117

Transfected allogeneic islet transplants.. 117
  1. Allograft survival.. 117
  2. Histology of kidneys harvested at 60 days.......................... 120
  3. Relationship between allograft survival and pre-transplant transgene expression.. 121

DISCUSSION.. 121
Optimizing the amount of DNA and gold particles used for transfection..... 121
Transfection control grafts... 123
Transfected allogeneic islet transplants.. 124

REFERENCES.. 141

VI. GENERAL DISCUSSION

BIOLISTIC TRANSFECTION OF INTACT ISLETS........................... 144
  1. Advantages... 145
  2. Limitations... 146

GENE THERAPY AND ISLET TRANSPLANTATION........................ 148
  1. Human CTLA4-Ig.. 148
  2. Human soluble Fas ligand.. 150

ACHIEVEMENTS OF THIS STUDY.. 152

FUTURE DIRECTIONS.. 153

REFERENCES.. 156

APPENDIX

DETAILS OF GENE CONSTRUCTION.. 160

LIST OF TABLES

TABLE	DESCRIPTION	PAGE
1-1	Immunomodulation protocols which have achieved prolonged islet graft survival.	31
1-2	Summary of transfection methods used with pancreatic islet tissue.	32
2-1	Efficacy of gene introduction into mouse islets.	61
2-2	Insulin response of islets to static glucose challenge following biolistic transformation.	62
3-1	Survival of islet allografts: BALB/c mice transplanted with 500 biolistically transfected CBA islets.	77
4-1	The effect of the number of islets exposed per biolistic blast.	96
4-2	The effect of different helium pressures per blast.	97
4-3	The effect of gold particle size.	98
4-4	Comparison of sample in hanging drop vs sitting drop.	99
5-1	The effect of the quantity of DNA and gold particles on islet damage and transfected gene expression.	131
5-2	Insulin levels of transfection control grafts.	132
5-3	Graft survival and insulin extractions of transfected islet transplants.	133
5-4	Histological appearance of long term allografts harvested at 60 days.	134
5-5	Relationship between allograft survival and pre-transplant expression.	135

LIST OF FIGURES

FIGURE	DESCRIPTION	PAGE
1-1	Mechanisms of allograft rejection.	33
1-2	The Two Signal Hypothesis of T cell activation.	34
2-1	Effect of helium pressure and number of islets per biolistic exposure.	63
2-2	Scanning electron micrograph of a biolistically transfected mouse islet.	64
2-3	Natural scanning electron micrographs of a biolistically transfected mouse islet at two different electron-accelerating voltages.	65
2-4	In vivo function of tranplanted biolistically transfected islets.	66
3-1	Blood glucose levels of mice transplanted with islets biolistically transfected with human CTLA4-Ig.	78
3-2	Photomicrographs of biolistically transfected CBA mouse islet grafts harvested from BALB/c recipients.	79
4-1	Principle of biolistic transfection procedure.	100
4-2	The effect of helium pressure on viability of blasted islets.	101
4-3	The effect of gold particle size on viability of blasted islets.	102
4-4	Reproducibility of biolistic particle acceleration onto cell strainer targets.	103
4-5	Reproducibility of biolistic particle acceleration onto Petri dish targets.	104
4-6	Patterns of gold particle bombardment with different tissue loading procedures.	105

FIGURE	DESCRIPTION	PAGE
5-1	Blood glucose levels of mice bearing transfected long-term allografts, harvested at 60 days.	136
5-2	Photomicrographs of histological sections from kidneys harvested at 60 days which were transplanted with allogeneic islets transfected with human CTLA4-Ig.	137
5-3	Photomicrographs of histological sections from kidneys harvested at 60 days which were transplanted with allogeneic islets transfected with human soluble Fas ligand.	138
5-4	Photomicrographs of histological sections from kidneys harvested at 60 days which were transplanted with allogeneic islets transfected with a combination of human CTLA4-Ig and human soluble Fas ligand.	139
5-5	Hypothetical scenarios of allograft and pancreatic insulin content in alloxan-induced diabetic mice.	140
A-1	Schematic of immunomodulatory molecules used in allotransplant studies.	161
A-2	Cloning strategy for human CTLA4-Ig.	162
A-3	Synthetic oligonucleotides used in synthesis of human CTLA4-Ig.	163
A-4	DNA sequence of the human CTLA4-Ig gene construct.	164
A-5	DNA sequence of the human soluble Fas ligand gene construct.	165

LIST OF ABBREVIATIONS

APC	antigen presenting cell
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
DAF	decay accelerating factor
DMEM	Dubellco's modified Eagle's medium
DTH	delayed-type hypersensitivity
DTT	dithiothreitol
FasL	Fas ligand
FasR	Fas receptor
G-CSF	granulocyte colony stimulating factor
ICAM	intercellular adhesion molecule
IFN-γ	gamma interferon
IL	interleukin
LFA	lymphocyte function-associated
MHC	major histocompatibility complex
PCR	polymerase chain reaction
PMA	phorbol 12-myristate 13-acetate
pNPP	para-nitro phenylphosphate
RLU	relative light units
SEM	standard error of the mean
TCR	T cell receptor
TGF	transforming growth factor
T_H	T helper cell
TMB	tetramethylbenzidine
TNF	tumour necrosis factor

CHAPTER I

GENERAL INTRODUCTION

DIABETES MELLITUS

There are currently upwards of one million individuals in Canada who suffer from diabetes mellitus, with the incidence steadily increasing. In fact, the Canadian Diabetes Association of Canada has termed this disease "the epidemic of the 21st century". Apart from the medical issues, the disease is also responsible for a major expenditure of increasingly scarce health care dollars.

Clinical disease generally falls into one of two categories: Type I (juvenile onset) insulin dependent disease and Type II (maturity onset) disease which may or may not require exogenous insulin. Although both have a genetic component, the pathophysiology of the two diseases is quite different. Type II disease is responsible for the majority of the disease burden, and is basically a metabolic syndrome. In contrast, Type I disease, the result of an autoimmune process, is less common but is the major cause of chronic childhood disease in the western world. This treatise will focus on Type I disease, and its treatment by islet transplantation.

The history of diabetes mellitus is a long one. As early as the first century A.D., Arataeus described the disease as "a melting down of the flesh and limbs into urine" (for an excellent historical review see (*1*)). From the middle of the nineteenth century autopsy evidence gradually accumulated which demonstrated that the disease was often accompanied by damage to the pancreas. More importantly, extensive pancreatic damage almost always resulted in diabetes. However, the link remained tenuous until 1889 when Minkowski and von Mering, at the University of Strasbourg, demonstrated that total pancreatectomy in dogs caused the development of diabetes. By then it was known that the pancreas had two functions. The digestive juices, released into the gut, were known as the pancreas' external secretion. An internal secretion, released directly into the blood stream, was linked to carbohydrate metabolism. In 1894, Watson Williams (Bristol) unsuccessfully attempted to treat a 15 year old diabetic child using walnut-sized grafts of sheep pancreas transplanted into the subcutaneous tissues (*2*). It was not until 1901, however, that the pathological connection between diabetes and the islets of Langerhans (which had been originally described by Paul Langerhans in 1869) was shown by Opie. Once the link between the pancreas and diabetes had been solidly established, the hunt for the elusive pancreatic extract began in earnest, and during the first two decades of the twentieth century many researchers used extracts to try to replace pancreatic function . The formal discovery of insulin is credited to Banting and Best, working with Macleod and Collip, in Toronto, Canada in 1921. Pharmaceutical companies were quick to see the therapeutic value of such a substance and rapidly developed commercially available injectable forms of insulin derived from animal sources. Although immune reactions to the xenogeneic protein did occur over time, they generally only meant that steadily increasing doses of the drug were required to maintain glycemic control. This problem has now been largely overcome by the availability of human recombinant insulin, where the gene for human insulin has been introduced into yeast and the 'human' protein is produced in large quantities.

Although the availability of injectable insulin revolutionized the treatment of diabetes mellitus, and had an enormous impact on the quality of life and life expectancy of afflicted individuals, it has not provided the ultimate answer. Infrequent intramuscular administration of a large bolus of exogenous insulin is a far cry from the physiologic state where a basal steady state is maintained with super-imposed rapid intravenous responses to increases in blood glucose levels. Although it was intuitively believed that tight control of blood glucose levels would result in lower disease complication rates, this was not formally demonstrated until the release of the results of the Diabetes Control and Complications Trial (*3*) in 1993. Various approaches have been used over the years to address the problem of intermittent dosing ranging from chemical formulations which resulted in longer action of the insulin, to pumps which provided constant infusion, to injection devices which allowed easier administration of multiple doses (insulin 'pens').

ISLET TRANSPLANTATION

1. Advantages.

Obviously, the best way to regain the normal physiological state of affairs including precise blood glucose control is to replace the ß cells which have been lost due to immune damage by healthy insulin-producing tissue. This could be achieved through transplantation of either an entire pancreas or isolated islets of Langerhans. Although whole organ transplantation has gained in popularity in several centres in the United States, it has not become well-accepted in Europe. Like any vascularized organ transplant, the surgical procedure is complex; it not only requiries access to a vascular supply but the organ also has to be anastomosed to the bladder to provide exocrine drainage. The morbidity associated with the procedure can therefore be considerable, and most of the transplanted tissue is functionally unnecessary. Although the rate of insulin independence at 1 year is 70%, long-term graft survival data in Type I diabetics may still be problematic (*4*). Therefore, it is still not clear whether the benefit of whole pancreas transplantation outweighs the morbidity of the procedure.

By contrast, in 1972 Ballinger and Lacy demonstrated the feasibility of islet isolation and transplantation in rodents (*5*). Although the in vitro isolation procedure of human islets is complex, the islet transplantation procedure is extremely simple. Once the umbilical vein has been cannulated, providing external access, the actual transplantation procedure is performed at the bedside where the tissue is simply infused into the liver bed. The greatest procedural complication is that of portal hypertension, requiring close monitoring of portal pressures during the infusion process.

Perhaps one of the greatest advantages of islet transplantation is that the isolation procedure provides an opportunity for in vitro treatment of the islets before transplantation into a recipient. A number of modalities have been used at both the molecular and cellular levels in order to influence the immune attack against the graft subsequent to transplantation. These various immunomodulatory strategies will be discussed in more detail below.

2. Past experience.

Clinical studies of islet transplantation began in earnest in 1974. Based on review of studies between 1990 and 1993, four characteristics were identified to be associated with long-term insulin independence (*6*). These included a pancreas preservation time of <8 h, infusion of >6000 islet equivalents per kilogram of body weight, liver as transplantation site , and immunosuppression with anti-T cell antibodies. When these criteria were met, 46% of patients had persistent C-peptide secretion at 1 year follow-up, although 83% of patients showed initial C-peptide levels >1 ng/mL. Of those, 29% were insulin independent for 1 year (*6*). Therefore, 30-40% of islet allografts unfortunately completely lost their function within the first two months following transplantation. In 1994, 28 adult islet allotransplants were performed, with C-peptide levels >1ng/mL for >1 month in 32% of cases, and insulin independence >1 week in only 7% (*6*). Obviously the issues of primary non-function and immune attack on the transplanted tissue remain largely unresolved.

3. Outstanding problems.

Consistent availability of large numbers of highly purified, viable human islets from cadaveric sources continues to be a problem. Donor numbers have, in fact, progressively decreased over the past several years. Commercial availability of a reproducible high grade collagenase has been an enormous frustration and barrier to further improvements in the field. Fortunately this problem is now being seriously addressed by some of the major enzyme manufacturers.

Allograft rejection of the transplanted islets also has to be overcome. Currently, this is achieved through chronic use of systemic immunosuppressive agents. For this reason islet transplants to date have either largely been carried out simultaneously with, or subsequent to the transplantation of another solid organ, most often a kidney. Immunosuppressive drugs such as Cyclosporine A (*7*), azathioprine (*8*) and corticosteroids (*9*) all have adverse effects on early islet graft survival. In addition, they all have toxic side effects and require close monitoring of blood levels in patients. Some of the recent immunomodulation approaches, such as the gene therapy approach proposed in this thesis, may mean that systemic immunosuppression can be circumvented. Other approaches to avoid allograft rejection are cellular immunodulation and immunoisolation techniques.

Another remaining barrier to long-term islet (as well as whole pancreas) graft survival in diabetes, is the destruction of the transplanted tissue by the autoimmune process, leading to ultimate recurrence of the disease. This phenomenon has been documented in cases of segmental pancreatic transplants between identical twins, where MHC incompatibility was obviously not a factor (*10*). Graft destruction by recurrent autoimmunity has also been shown in a case of a human islet allograft transplantation (*11*). Although the following study is limited to addressing immunity to allogeneic tissue, with further understanding of the autoimmune process it may be possible to address the autoimmunity problem through similar gene therapy immunomodulatory approaches.

CELLULAR IMMUNOMODULATION TO OVERCOME ISLET ALLOGRAFT REJECTION

As early as 1975, it was shown by Lafferty et al. that survival of thyroid allografts in mice could be prolonged by first subjecting the tissue to 7 day culture at 37°C (*12*). This was followed by a number of studies which showed that various types of tissue treatment could cause a prolongation of islet allograft survival. A brief overview of the protocols which have been used successfully in a variety of systems is presented in Table 1-1. They are all based on the assumption that MHC Class II positive, costimulatory antigen presenting cells (APCs) which were present within the graft as passenger leukocytes were the main source of graft immunogenicity (*13*), and that inactivation or removal of these passenger APCs caused a decrease in immunogenicity of the engrafted tissue. In other words, it appeared that the major pathway responsible for the triggering of allograft rejection was the direct, donor APC-dependent pathway. This is in contrast to the indirect, recipient APC-dependent pathway, which seems to play a more major role in the process of xenograft rejection (*14*).

As shown in Table 1-1, similar immunomodulation protocols have been utilized in both the murine and rat rodent systems. However, it appears that in the case of the rat model, most treatments have also required some degree of immunosuppression in the recipients in order to achieve a significant prolongation of the islet graft survival (*15-18*). Unfortunately, many fewer studies have been carried out using large animal models, due mainly to the technical problems and the cost of conducting these experiments. Again, low dose immunosuppression seemed to be an additional requirement in the canine model (*19, 20*).

The concept of transplantation of xenogeneic tissue has recently become much more popular. Although success has been achieved in rodent systems, very few "human" studies have been performed. A small number of xenograft studies using human islets and murine recipients have been reported as outlined (*21-23*).

A few attempts have been made by Scharp et al. (*24*) to transplant human islets that have been cultured at 24°C for 7 days to human recipients. Unfortunately, although initial C-peptide production was detected, all grafts were rejected by 2 weeks. An interesting model system to examine the effects of immunomodulation protocols on human islets was described by London et al. (*25*), where a "human reconstituted" severe combined immunodeficient mouse was proposed for use as a recipient.

An ideal method to achieve the desired degree of immunomodulation has yet to be discovered, and the study and development of immunomodulation regimens is still being pursued today.

THEORY OF GENE THERAPY (MOLECULAR IMMUNOMODULATION) IN ISLET TRANSPLANTATION

For islet transplantation to become a generally accepted form of therapy for otherwise healthy, type I diabetic patients, a method must be found to successfully transplant the insulin-producing tissue without any requirement for ongoing systemic immunosuppression. Gene therapy represents an important advance which will move us closer to this goal.

With the advent of recombinant DNA technology and methods to transfer additional new genes into eukaryotic cells, it has become possible to perform gene therapy on isolated islets ex vivo, before their transplantation into a diabetic recipient. In this regard, isolated islets have a distinct advantage over the intact pancreas or other solid organs, since islets can be easily manipulated in tissue culture and can be maintained for some time in vitro.

The basic hypothesis underlying this approach is that by introducing genes which encode immunosuppressive or 'immune-blocking' molecules into isolated islets, the transplanted tissue can be protected from immune rejection. The assumption is that local immunosuppression can be acheived within the graft, while leaving the rest of the immune system intact. Once established, besides eliminating the untoward effects of systemic immunosuppressive agents, this technology may also have the added advantage of being less expensive than conventional immunosuppressive therapy.

Two main questions must be considered when applying gene therapy to the problem of preventing immune rejection of transplanted islets:

1) What are the most suitable methods for introducing new genes into islets?
2) What gene or genes are most useful in preventing the allograft immune response?

GENERAL TRANSFECTION METHODS

The process of introducing new genes into cells or tissues is known as DNA-mediated gene transfection, or more simply as 'gene transfection'. Gene transfection methods can be divided into two major categories: 1) non-viral methods, and 2) viral methods. The former category uses purified, 'naked' DNA, which may or may not be complexed with some other macro-molecule or macromolecular complex, whereas in the latter category the DNA is packaged into an infectious recombinant viral particle.

Non-viral methods of transfection may be chemical approaches which rely on normal cellular processes for uptake, or they may be physical, wherein some deliberate manipulation is carried out on the cell to force DNA uptake. Typically, chemical methods cause little or no damage to the cell, whereas physical methods can destroy some or even many of the cells being transfected. Chemical methods generally involve co-precipitation or binding of DNA into macro-molecular complexes, such as crystals of calcium phosphate, complexes of cationic polymers such as DEAE dextran, or small liposomes with a positive surface charge which bind the negatively charged DNA. The macromolecular complexes containing the DNA either settle passively onto the cytoplasmic membrane of the target cells, or bind through charge interactions, and are taken up largely by endocytosis or pinocytosis. Alternatively, physical methods involve some type of additional cell insult, and therefore a certain degree of cell damage, to achieve uptake. Electroporation and biolistics are examples of these kinds of methods.

Viral methods use recombinant viral particles to carry the foreign DNA into the cells during the course of cellular viral infection. Although the engineered viruses maintain their infectivity, they have been engineered to render them replication deficient. Each of these transfection methods has its own set of limitations and potential problems and further specific discussion of each appears in the following section.

1. Non-viral methods of gene transfer.

DNA co-precipitation methods are still widely used to transfect eukaryotic cell lines. The methods are inexpensive, simple to perform, and transfection efficiencies can be reasonably high depending on the cell line used. A brief shock with DMSO or glycerol, and/or the presence of chloroquine can enhance the process of uptake and transformation. A key point is that the ingested DNA travels first into the phagolysosomal compartment of the cell, and if acid nucleases are present, the DNA will be largely or completely destroyed. Experience suggests that the reason that many established (i.e. immortalized) cell lines which have been carried for a long time in tissue culture transfect efficiently with chemical methods is because they have reduced levels of acid nucleases. In contrast, most ex vivo tissues or primary cell lines (i.e. taken directly from a live animal, and not immortalized) are very difficult to transfect by chemical methods.

Lipofection can be performed using either monovalent or multivalent cationic lipids (e.g. Lipofectin and Lipofectamine, respectively; GIBCO/BRL, Gaithersburg, MD,USA).

The multivalent forms have consistently been found to achieve higher transfection rates. The exact nature of the interaction between the negatively-charged DNA and the cationic lipid micelle is still not completely understood, but it is generally accepted that the majority of the DNA/liposome complexes are delivered into the cell through endocytosis (*26*). Once internalized, the membrane rupturing capability of cationic liposomes is thought to destabilize the endosomal membranes resulting in cytoplasmic release of the DNA/liposome complexes. This ability to rupture the endosome may result in protection from the actions of its degradative enzymes and be the explanation behind the higher transfection efficiencies obtained with this methodology than with the co-precipitation methods. However, it is still difficult to transfect primary tissues with high efficiencies using lipofection. It is also a relatively expensive procedure when using commercially prepared cationic liposomes.

A more recently developed chemical method of transfection involves the use of polylysine-ligand/DNA complexes (*27*). A targeting ligand is chemically coupled to positively-charged polylysine molecules, which in turn interact with negatively-charged DNA. The targeting ligands can include molecules such as transferrin or asialoglycoproteins, which bind to their specific receptors on the cell surface and stimulate receptor-mediated endocytosis. The advantage of this system is that it allows specific targeting of the DNA to the tissue. As with the co-precipitation methods, however, the DNA is subject to endolysosomal degradation. To circumvent this problem, chemically-inactivated adenovirus has been used as a targeting ligand. The complex is internalized in a similar fashion, but the virus has an endosomolytic action which ruptures the endosomal membrane, thus avoiding degradation of the DNA. This technology is being further improved through the development of more superior ligands. For example, microorganism-derived peptides which lead to endosomal lysis are being investigated in order to develop virus-free, targeted transfection systems (*28*). The future should see the development of more specific cell-targeting, higher efficiencies and longer expression times with the addition of newer elements to the system.

With the exception of DMSO or glycerol shock, chemical gene transfer methodologies have little detrimental effect on the target tissue, since the DNA is basically taken up through a natural cellular process. Once within the cytosol, the DNA can be transported to the nuclear compartment where it exists almost entirely in an episomal form, and is therefore eventually degraded by the cell. Gene expression is thus transient, usually in the order of weeks to months, being affected also by the rate of division of the cells and the resultant dilution of the foreign gene.

As previously mentioned, non-viral gene transfection can also be performed by physically damaging the cells to enhance DNA uptake. In the case of electroporation, the cells are suspended in a cuvette in a solution containing the DNA. Upon momentary exposure to a high electrical field, it is hypothesized that small pores are transiently formed in the cellular membrane (*29*). Consequently, DNA-containing fluid flows into the cell. Although this procedure can be highly efficient, including application to primary tissues in some cases , the exposure to the electrical shock does damage a significant number of cells. A totally different approach is taken with biolistic transformation, or the gene gun (*30*). Here, DNA is precipitated onto gold or tungsten particles which are approximately 1.0 - 4.0 μm in diameter. With the currently available technology, the microparticles are accelerated to high velocity using high pressure helium gas, and shot into the cells. The rents torn in the cellular membrane are re-sealed by the cell. The inert particles remain in the cytosol, where the DNA is solubilized. This is probably the most harsh process of all of the outlined methods, with a large proportion of the cells being damaged from the effect of the helium shock wave. The advantage of the biolistic transfection system is that it can be used on virtually any cell type, including primary tissues. Disadvantages are the sometimes low transfection rates, and problems with reproducibility, related both to the efficiency and stability of the DNA/gold precipitation process and the technical limitations of the apparatus. Again, in both the case of electroporation and biolistics, the DNA is mainly in an episomal form and expression is therefore transient.

2. Viral methods of gene transfer.

Viral-based methods of transfection are currently very popular due to the high transfection efficiencies that can be obtained. The DNA of choice is inserted into a viral genome, which is then surrounded by viral packaging to form an infectious viral particle. During the process of cellular infection, the passenger DNA is transferred into the cell, a process referred to as transduction. Depending on the virus used, the DNA can either end up in an episomal form or integrated into the host chromosome. Several different viruses can be used as vectors, including pox-, herpes-, adeno-, adeno-associated (parvo) and retroviruses. Since the pox viruses are not replication deficient, they ultimately cause lysis of the target cells. For this reason, they are not widely employed for gene therapy. The latter four viruses, however, have been manipulated so that they are replication deficient despite being able to infect the target cell. Not surprisingly, however, each has associated problems. In addition, viral techniques can be costly due primarily to the expense of virus generation/preparation and biosafety requirements.

Adenoviruses are non-enveloped lytic DNA viruses. The most widely used replication-deficient recombinant adenoviral vectors have been engineered so that the E1 and/or E3 regions have been deleted. Adenoviral vectors have become very popular for a number of reasons (see (*31*) for review). High titre viral stocks can generally be produced although this is somewhat labour-intensive. Adenoviruses have a broad host species spectrum. They are capable of transfecting a wide range of target cell types, and generally do so with extremely high efficiencies. This is particularly true for epithelial cells since they are the virus' natural targets. The corollary of this, however, is that the DNA can be rapidly lost through epithelial shedding. Perhaps the greatest advantage of adenovirus is its ability to transfect primary, resting or non-dividing cells. Disadvantages of adenovirus include the following. Gene expression is transient since the DNA remains essentially in an episomal form and is therefore ultimately degraded after a period of weeks. In addition, there is now good evidence that the adenoviral proteins produced within the cells are immunogenic (*32*). An immune response is therefore mounted against them when used in vivo. However, new recombinants with further deletions of the viral genome are being developed (e.g. E2a region deleted) which may help to reduce this problem (*33*). In addition, re-insertion of the E3 portion may in fact reduce inflammation caused by the virus.

All of the chemical, physical and viral transfection methods previously discussed do not result in long-term gene expression because the DNA does not become permanently integrated into the host chromosome. In contrast, both adeno-associated viruses and retroviruses have this capability. Theoretically, gene expression should therefore be permanent. However, due to other problems associated with each of these vectors their use has been somewhat limited.

Retroviruses have a complex life cycle. The replicative cycle includes packaging of the RNA into virions which enter the target cell. The RNA undergoes reverse transcription to form a DNA provirus which is then stably integrated into the host genome (*27*). Advantages include a wide host range and stable integration. However, there are several disadvantages. In general, it is difficult to obtain high titre viral stocks of the retroviruses. In addition, the retroviral vectors which are available to date are derived from the Moloney murine leukemia virus, which means that integration is largely limited to proliferating cells. Perhaps most disappointing, however, is the fact that permanent gene expression has not proven to be the case with retroviruses (*34*). Although integration occurs, expression of the foreign gene wanes over time. Although the reasons are not completely understood, base methylation of the viral promoter/enhancer element by the eukaryotic host probably plays a role (*34*). While chromosomal integration is thought to be desirable on the one hand, it also poses serious concerns. In the case of retroviruses, integration occurs randomly within the chromosome, and there is the possibility of insertion within a protooncogene with the resultant serious consequences. As a result, these vectors have only been employed for gene therapy in cases where the alternative outweighed this risk, and the incidence of tumour formation will be revealed over time. Finally, the possible creation of replication-competent retroviruses remains a persistent threat in terms of clinical applications. There has been at least one report of the development of a T cell neoplasm in primates which is thought to have originated from contaminating replication-competent recombinant retroviruses (*35*). On the other hand, rapid advances are being made to improve these vectors through further engineering to minimize or eliminate the current problems associated with them.

In contrast to retroviruses, infection of non-proliferating cells is possible with adeno-associated viruses (parvoviruses), although the chances of chromosomal integration under these circumstances may be lower (*36*). There have also been several reports of persistent episomal forms with recombinant adeno-associated viruses (reviewed in (*37*)). These viruses were thought to have the advantage of inserting at specific sites within the host chromosome (*38*), but this has only proven to be true with wild-type virus, not with the recombinant forms. Therefore, the same concerns regarding insertional mutagenesis apply to them as to the retroviruses, with respect to their use as gene therapy vectors.

Herpes simplex virus can also be employed as a viral vector although its use is less common. This virus is a double stranded DNA virus of which large segments can be deleted, allowing the insertion of very large fragments (up to 30 kb) of foreign DNA. Like the adenoviruses, they have been made replication-deficient and remain in an episomal form. They are capable of infecting non-dividing cells and their main advantage lies with their capability for gene therapy within the nervous system (*27*).

In summary, a wide range of methods, both viral and non-viral, is available to transfect eukaryotic cells, each with its own advantages and disadvantages. For any given series of experiments it is therefore important to carefully evaluate the long term goal of a study and to consider the type of target cell to be used, the required transfection efficiency, and whether transient gene expression will be sufficient to give a suitable readout.

TRANSFECTION EXPERIENCE RELATED TO ISLET TRANSPLANTATION

Three possible approaches can be considered within the context of using transfected cells in islet transplantation. Transfection could be performed on dissociated islet cells, intact islets or on some kind of unrelated cell which would be co-transplanted along with the islets. Although the number of studies is still small, each of these possibilities has been investigated. A summary of the studies performed to date appears in Table 1-2. Note that this is a summary of all of the transfection procedures attempted and not all of them have been successful.

In general, primary cells are much less receptive to transfection than cultured cell lines, particularly to chemical methods. As previously mentioned, this may be due to differences in acid nuclease levels. More specifically, however, intact islets present an additional difficulty related to their architecture. An islet is a spherical cluster of cells, with many cells buried within the core so that their membranes are relatively inaccessible. In addition, the entire structure is surrounded by a layer of connective tissue which further limits membrane accessibility. In the study by Saldeen et al. (*39*) intact islets from several species were found to be difficult to transfect using any of the chemical methods. We have also experienced very low transfection rates using Lipofectamine (GIBCO/BRL) on intact islets. Conversely, transfection rates are considerably improved, on the order of 20 to 50 times, if the islets are first dispersed into single cells (*39*). Interestingly, the same study demonstrates that there is a species difference as to which transfection method is optimal. Electroporation has also been successfully used to transfect single cells from dispersed islets (*40-43*). In these cases, islets from either obese, hyperglycemic mice or fetal Sprague-Dawley rats were dispersed using trypsin/EDTA, and the resultant single cells were electroporated and then cultured. Lipofection has been performed on similar cell preparations using locally prepared liposomes (*41*). Unfortunately, the insulin-secreting capacity of the cells following the transfections was not assessed.

Perhaps more relevant to the transplant setting are the successful transfection studies performed on intact islets. In contrast to the studies mentioned above which failed to achieve successful transfection using lipofection, one group has reported success (*44*). Southern blots indicated successful uptake of the DNA, although there was no direct evidence of gene expression by these islets. While the transfected islets were transplanted into mice, analysis of their insulin-secreting function was not done since numbers were used which would not result in reversal of hyperglycemia.

We have not obtained efficient transfection of islets in suspension using lipofection, but we have successfully transfected intact islets using biolistics (*45*). Due to the nature of the transfection process, this method is able to overcome the architectural barriers of the intact islet. However, the process is damaging, with only 50-70% of the initial number of islets being recovered. Those islets that do survive the transfection process show that about 3% of the total cells are expressing the exogenous gene, with each of these cells containing multiple gene copies since each gold particle holds more than one molecule of DNA. Furthermore, transplanted biolistically-transfected islets functioned in vivo to reverse diabetes. We have recently improved the process such that we can now obtain luciferase activity encoded by the foreign DNA in the order of 140 times greater than that which we published previously (see Chapters II and IV).

By far the most efficient transfection process for intact islets is infection with recombinant viruses. Newgard's group pioneered the method of adenoviral infection in intact islets, achieving gene transfer into 70% of islet cells, with expression persisting up to 1 month in culture with no adverse effects on in vitro insulin-secreting capacity (*46*). Similar results have been obtained by others, including good in vivo function following transduction (*47-49*). Again, this system is only useful for short-term expression with one time exposure since immune responses to adenoviral antigens have been documented (*32*).

More recently, successful transfection of both intact islets and dispersed single cells has been achieved using a defective Herpes simplex virus-1 amplicon vector, with approximately 80% of islet cells transfected (*50*). Presently available retroviral vectors have been tried unsuccessfully on islets, failure presumably being due to the extremely low percentage of proliferating cells present (*47*).

Since gene transfer into intact islets has been either relatively inefficient or associated with viral immunogenicity which may result in graft clearance, another approach is to consider co-transplantation of an independent transfected cell along with non-transfected islets. This model has been employed using a transfected muscle cell line (*51*). The obvious problems associated with this concept are tumour formation and overgrowth of the graft by the accompanying immortalized 'nurse' cells. Ideally, one would wish to use a non-motile, non-proliferating cell which is efficiently transfected using a non-viral method. A study which utilized co-transplanted primary myoblasts transfected by lipofection and selected for gene expression appears to fulfill these criteria (*52*). However, with respect to an intra-islet passenger leukocyte (e.g. a donor antigen presenting cell), it may be advantageous to have protein production centrally from an islet cell, rather than from a peripheral location. Due to further improvements in transfection techniques, the number of studies using both transfected islets and co-transplanted transfected cells is rapidly increasing.

CANDIDATE MOLECULES FOR IMMUNE INTERFERENCE BY GENE THERAPY IN ALLOGRAFT REJECTION

The activation of recipient CD4+ T helper (T_H) lymphocytes by donor antigen presenting cells is thought to be a critical initial step in the process of allograft rejection (*53*). Classically, CD8+ cytotoxic lymphocytes (CTLs) have been thought to inflict the majority of the tissue damage (*54*). However, a recent report challenges the paradigm that the CTL is the major effector cell responsible for allograft destruction (*55*). Furthermore, it is now known that the magnitude of a CTL response is significantly diminished in the absence of CD4+ T cell help (*56*). Although the relative contribution of each effector mechanism may still be an area of controversy, it is generally accepted that several additional processes are involved in allograft rejection, including delayed type hypersensitivity (DTH), CD4+ cell mediated damage and humoral responses (*53*). Figure 1-1 outlines the mechanisms involved and their respective interactions (*53*). With expansion of our knowledge of these events at a molecular level, it is now possible to attempt to disrupt specific key steps involved in the rejection process. The advantage of using a gene therapy approach as opposed to systemic agents is that the specific immune interference could be limited to the site of the graft.

In general, immune interference could be attempted in order to achieve three basic goals: the prevention of T_H cell activation, the removal or neutralization of effector cells, and/or the reprogramming of the overall immune response through the production of immune-modulating cytokines. Each of these concepts is briefly discussed below.

1. The prevention of T_H cell activation.

It is now generally accepted that two signals are required for T cell activation (*57*). Signal 1 occurs with engagement of the antigen-specific T cell receptor by either foreign antigen in self-MHC or by a foreign MHC molecule. Signal 2 is a co-stimulatory signal that is provided by metabolically active antigen presenting cells (APCs). In islet grafts, these intra-islet APCs have been termed passenger leukocytes (*13*) and a number of in vitro manipulations have been used effectively to remove or damage them before islet transplantation. This type of immunomodulation has been discussed earlier in this chapter. Rather than APC removal, however, an attractive concept is simply to block the co-stimulatory signal provided by the donor APCs, using a molecular immunosuppressive strategy.

The most important effect of the introduction of a gene encoding a co-stimulatory blocking protein would be the blockade of T_H cell activation. A diagrammatic representation of such an approach appears in Figure 1-2. Interference at the level of the APC:T_H lymphocyte interaction (and subsequent T_H activation) should in turn have a deleterious effect on all of allograft rejection effector mechanisms (see Figure 1-1). If ongoing prevention of activation required continuous blockade, permanent transfection would be a necessity, which is not yet possible for islets with currently available techniques. However, the presence of Signal 1 in the absence of Signal 2 has resulted in long-term lymphocyte hyporesponsiveness in vitro (*58*). If similar events occur in vivo, blockade of the co-stimulatory signal would theoretically only be required in the initial period following transplantation. It follows that transient transfection should be sufficient to achieve this initial blockade.

B7:CD28 interactions

Currently, the B7 (APC):CD28 (T cell) interaction is thought to be one of the principal co-stimulatory signals involved in T cell activation (*56*). The CD28 molecule was discovered to be a member of the immunoglobulin superfamily, and to contain a single extracellular V-like domain (*59*). It was subsequently discovered to have an accessory (co-stimulatory) function during T cell activation (*60*). Further study showed that the CD28 molecule adhered to 'B cell activation antigen', or B7, which is also a member of the immunoglobulin superfamily (*61*). Further to this, P.S. Linsley reported in 1991 that

CD28 was the primary receptor for B7 on activated peripheral T cells, and that the two molecules bound to each other in the absence of other accessory molecules. The K_d between B7 and a CD28-Ig fusion protein was calculated to be approximately 200 nM (*62*). The same study also demonstrated that theB7:CD28 interaction was co-stimulatory for T cell activation since it led to the proliferation of T cells and increased levels of IL-2 messenger RNA transcripts (*62*).

CTLA4

In 1987, Brunet et al. discovered a new molecule, CTLA4, which was homologous to CD28, when performing differential screening of a murine cytolytic T cell cDNA library (*63*). Transcripts for CTLA4 were then found in other T cell populations which had cytotoxic activity (*63, 64*). In 1988, the gene for human CTLA4 was cloned (*65*) and was mapped to the same chromosomal region as CD28 (*66*). Interestingly, significant homology exists between the CD28 and CTLA4 molecules, particularly in the cytoplasmic regions (*64, 65*). In addition, the cytoplasmic domains of the murine and human forms of the CTLA4 molecule are identical, suggesting an important signaling function (*65*). It has been shown that CTLA4 mRNA is present in only low levels, if at all, in naive T cells, but can be detected in both CD4+ and CD8+ cells, and in essentially all T-cell clones, following activation (*67, 68*).

B7:CTLA4-Ig interactions

In 1991, P.S. Linsley began his now extensive study of the CTLA4 molecule. As in previous studies of the CD28 molecule (*62*), a fusion protein was constructed which consisted of the extracellular portion of the human CTLA4 molecule linked to a human IgCγ1 tail which had had the disulfide bridges in the hinge region disrupted by cys to ser substitutions (*69*). The calculated K_d of CTLA4-Ig for B7 was approximately 12 nM, in the order of 20 times greater than that of CD28-Ig (K_d of 200 nM). This value compares favourably with higher affinity monoclonal antibodies (K_d 2-10,000 nM (*70*)), K_d values of integrin receptors and their ligands (10-2,000 nM (*71-73*)), interactions between CD2 and LFA-3 (400 nM, (*74*)), and the binding of soluble alloantigen to the TCR of a murine T cell hybridoma (approximately 100 nM, (*75*)). Further, CTLA4-Ig inhibited a mixed lymphocyte reaction in a dose-dependent manner (*69*). The soluble CTLA4-Ig molecule binds to the various isoforms of B7 present on APCs (*76*), thereby preventing their interaction with both CD28 and CTLA4 receptors on T cells . Stable transfectants of mammalian cells were subsequently made, which allowed production of large amounts of the CTLA4-Ig fusion protein (*69*).

Simultaneous reports appeared which extended the in vitro immunosuppressive effects of CTLA4-Ig to the in vivo situation. The use of intravenous injections of CTLA4-Ig resulted in long-term islet xenograft survival (*23*), and suppression of antibody responses to both sheep red blood cells and a soluble protein antigen, keyhole limpet hemocyanin (*77*). Several studies have demonstrated prolonged survival of rat heterotopic cardiac allografts when this molecule has been administered intravenously around the time of transplantation (*78, 79*). Similar findings have been found in mouse models (*80, 81*), and long-term donor-specific tolerance could be induced (*81*). It has also been shown that CTLA4-Ig inhibited alloantibody responses to repeated blood transfusions in rats (*82*). Prolonged allograft survival has also been reported using CTLA4Ig in combination with other immunosuppressive strategies in several systems. CTLA4Ig combined with donor-specific blood transfusion (*83, 84*), low-dose cyclosporine A (*85*) or anti-CD4 monoclonal antibody (*86*) has been successful in the rat heterotopic cardiac allograft system. The latter combination also produced indefinite survival of rat small bowel allografts and tolerance to subsequent heart allografts (*87*). Using a rat lung allograft model, the combination of CTLA4-Ig, single-dose cylcosporine A and donor-specific transfusions resulted in the most prolongation, but long-term acceptance was not achieved (*88*). Similar approaches have been taken in murine cardiac allograft models, with prolonged allograft survival being achieved with a combination of CTLA4-Ig and donor bone marrow (*89*). CTLA4-Ig plus an anti-CD4, anti-CD8 and anti-LFA-1 monoclonal antibody led to excellent mouse myoblast survival for > 1 month (*90*). Finally, a group has recently produced a form of CTLA4-Ig which has an IgM, rather than IgG tail which means that it exists in a pentameric form (*91*). When combined with sub-therapeutic doses of FK506, this molecule led to significant survival of mouse heterotopic, nonvascularized cardiac grafts. In summary, there is a substantial literature which indicates that systemic CTLA4-Ig can lead to prolongation of allograft survival in several systems.

Simply incubating mouse islets with the murine CTLA4-Ig protein in vitro before transplantation also resulted in long-term graft survival in 42% of allografts (*92*). In this case, there is no CTLA4-Ig directly administered to the mouse. These results again suggest that the direct, donor APC-dependent mechanism plays a significant role in allograft rejection. Co-transplantation of stable transfectants of a syngeneic murine muscle cell line along with mouse islets also conferred immune protection, allowing prolonged islet allograft survival (*51*). We have biolistically transfected mouse islets with the cDNA encoding for human CTLA4-Ig and achieved long-term survival in 50% of allografts (*93*). Further prolongation of allograft survival was obtained by the same group when an additional co-stimulatory molecule blocker, anti LFA-1, was used along with the transformed co-transplanted cells (*94*).

To reiterate, the rationale for all of these studies is that the soluble, higher affinity CTLA4-Ig molecule binds to B7 molecules present on the donor tissue, thus preventing interaction of these molecules with the lower affinity CD28 molecules on recipient T cells. Antigen-specific T cell activation is thus theoretically prevented. It is important to note that in all of the studies performed using CTLA4-Ig systemically (see above), there have been no adverse effects reported in terms of toxicity to the recipient.

Other potential interactions for targeting

Although we have limited our approach solely to the CD28:B7 interaction, there are several other lymphocyte:APC interactions whose disruption could potentially lead to prevention of T cell activation. It has been shown that blocking CD2:LFA-3 interactions using a soluble 'LFA-3-Ig' molecule can block T cell activation (*95-97*), and that CD2:LFA-3 signaling can reverse human alloantigen-specific clonal anergy (*98*). Other investigators have shown that CD40:CD40 ligand signaling is important for T cell activation (*99, 100*), that this activation pathway plays a critical role in allograft rejection (*101*), and that by treating mice with allogeneic lymphocytes and antibody to CD40 ligand, pancreatic islet allografts (and in some cases even skin grafts) can be accepted long-term (*102*). Finally, monoclonal antibody blockade of ICAM-1:LFA-1 interactions alone have also been shown to allow acceptance of mouse cardiac allografts, and the transplanted mice show donor specific tolerance to allogeneic skin grafts (*103*). Other authors have argued that blockade of the ICAM-1:LFA-1 signaling pathway results in immunosuppression without inducing alloantigen-specific anergy (*104*). Larsen and colleagues reported that they could achieve long-term acceptance of mouse skin and cardiac allografts by blocking both the CD40 and CD28 pathway (*105*). It is only a matter of time before many of these molecules, both alone and in combination, will be tested through transfection studies.

2. Removal or neutralization of effector lymphocytes.

An alternative approach to interruption of T cell activation, is to remove or inactivate cells which have become activated, in order to prevent their destructive effector function. One of the advantages of such an approach is that activated T cells would be targeted for destruction regardless of whether they had been activated by donor APCs (direct pathway) or recipient APCs presenting shed donor antigens (indirect pathway). An example of such a strategy would be the introduction of Fas ligand, in the hopes of causing apoptosis of activated, Fas receptor-expressing target lymphocytes. This system is discussed in detail below.

FAS receptor

The Fas receptor and complementary Fas ligand system has been discovered and characterized relatively recently. The discovery of what is now known as Fas receptor came about as the result of investigations involving apparent inconsistencies in the cytotoxic actions of tumour necrosis factor (TNF). The authors isolated a murine IgM monoclonal antibody called anti-Fas antibody, which was cytotoxic to a number of human cell lines (*106*). Using this antibody, they were subsequently able to identify a unique membrane protein called Fas antigen (*106*). Another group independently isolated a murine IgG3 monoclonal antibody called anti-Apo-1, which induced tumour regression in a particular B cell lymphoma line called BJAB (*107*). The gene for Fas antigen (receptor) was subsequently isolated by expression cloning from a cDNA library prepared from cells with high levels of Fas expression and consequent susceptibility to anti-Fas antibody toxicity (*108*). The gene encoding Apo-1 was cloned shortly thereafter, which established that it had the same identity as Fas antigen (*109*). The molecule is now generally known as Fas, Fas receptor, or CD95. Further study localized the gene to chromosome 19 in mice and chromosome 10 in humans (*110*).

Fas receptor is a Type I protein (meaning that it has an extracellular amino terminus, a transmembrane domain and a cytoplasmic carboxy terminus) and the mature protein is composed of 325 amino acids (*108*). A soluble, truncated form of Fas receptor (sFasR) which lacks the transmembrane region has also been identified, the result of alternate mRNA splicing (*111*). Fas receptor is a member of the TNF receptor family, and has significant homology to the TNF receptor in the cytoplasmic region. Mutational analysis revealed that the 70 amino acid region which was homologous between the two molecules was necessary and sufficient for transduction of an apoptotic signal (*112, 113*).

Fas receptor is constitutively and abundantly expressed by a variety of tissues including mouse thymus, heart, lung, liver, kidney and ovary (*110*). However, the same authors found human thymocyte Fas expression to be weak. Fas receptor is also highly expressed on activated mature lymphocytes (*107*). Further, Fas expression could be upregulated by IFN-γ in various cell lines (*106, 108, 110*), or IFN-γ in combination with TNF-α in human B cells (*114*).

Fas ligand

Fas ligand, the complementary molecule which binds Fas receptor, was identified by screening cell lines using fusion proteins composed of an IgG tail joined to either the extracellular region of the Fas receptor or TNF. It was found that the former molecule blocked the killing of Fas-expressing targets by a particular cell line called PC60-d10S (*115*). The authors were then able to purify the Fas ligand molecule from solubilized membrane fractions from a subline of PC60-d10S cells. The rat, murine and human genes were subsequently cloned, and localized to chromosome 1 in both humans and mice (*116-118*).

In addition to the protein isolated from solubilized membrane fractions, the Fas ligand molecule was isolated from supernatants of Fas ligand-expressing cell cultures. The membrane-bound and soluble forms were found to have equivalent biological activity (*115, 116*). Since the gene has no signal sequence, the soluble form of the protein is thought to arise through proteolytic cleavage of the membrane-bound form (*119*). Fas ligand has been characterized as a Type II protein (meaning it has a cytoplasmic amino terminus, a transmembrane region, and a extracellular carboxy terminus) and the mature protein is composed of 281 amino acids (*115*). There is 77% homology between the murine and human molecules and they are functionally interchangeable (*119*). In addition, significant homology with other members of the TNF family exists (*118*).

In contrast to the widespread expression of Fas receptor, constitutive Fas ligand expression is limited. It has been found on germ line cells (*120*), with abundant expression in mouse and rat testes (*116*). Interestingly, Nagata's group has not been able to find expression in human testes (*119*). Fas ligand is expressed within the anterior chamber of the eye (*121*). It is also present on activated T cells, but no Fas ligand-encoding mRNA could be found in B cells, macrophages, fibroblasts or endothelium (*122-124*). Further, expression on T lymphocytes can be rapidly induced by activation with PMA (phorbol 12-myristate 13-acetate), ionomycin or TCR engagement.

FasR:FasL interactions

Functional studies using mouse cell lines transformed with human Fas died by apoptosis within five hours following treatment with Fas ligand (*109*). Similar results have been obtained with Fas ligand-transformed COS cells and Fas receptor-expressing targets (*116*). The cell death caused by cross-linking of the Fas receptor has all of the hallmarks of apoptosis including the characteristic nuclear and cytoplasmic condensation and DNA fragmentation (*107*). The conclusions which can be drawn from these studies, are that Fas receptor transduces an apoptotic signal and that anti-Fas antibody and Fas ligand act as agonists. Isolated single, activated T cells have also been found to undergo apoptosis, indicating that one cell has both the stimulatory and effector mechanisms present to induce cellular 'suicide' (*125*). However, in other circumstances, Fas receptor can function in transduction of activation signals (*126*).

Based on experimental results to date, the Fas receptor/ligand system has a number of apparent functions. Firstly, the system provides an alternate CTL killing mechanism to the classically-described perforin system. While calcium is required for granzyme/perforin-dependent killing, in the absence of calcium, cytotoxic activity still remains and is attributed to the Fas/Fas ligand system (*127*). In addition, perforin knock-out mice retain their cytotoxic capacity towards Fas receptor-expressing cells (*128, 129*).

Secondly, the Fas receptor/ligand mechanism has also been postulated to play an important role in immunologic homeostasis. This conclusion has largely been based on findings in Fas receptor-deficient (lpr) and Fas ligand-deficient (gld) mice. Both strains develop lymphoproliferative disorders accompanied by lymphadenopathy, spenomegaly, and produce large quantities of antibodies including autoreactive anti-DNA and rheumatoid factor. They develop arthritis and nephritis and ultimately succumb to premature death around the age of 5 months (*117*). However, positive and negative selection in the thymus of lpr mice is apparently normal, suggesting that the Fas-mediated system of apoptosis is not involved (*130, 131*). This has led to speculation that the Fas/Fas ligand system is instrumental in the maintenance of peripheral self tolerance (*117, 132*). Perhaps the role of this system in the periphery is the deletion of self reactive clones which have escaped thymic negative selection, and the containment of activated T cell clones following antigenic stimulation.

A third proposed function is the maintenance of the immune-privileged nature of certain anatomical sites and tissues. Some examples of such sites include testis, ovary, eye and brain. Although the presence of an anatomical barrier may play a role in some cases, it is not the sole explanation since lymphocytes are not sequestered from all immune privileged tissues (*133*). It has been found that some of these sites, such as the cells in the anterior chamber of the eye and the testis, express Fas ligand constitutively (*120, 121*). In addition, the avoidance of graft rejection of so-called immune-privileged tissues (sertoli cells) when ectopically transplanted across allogeneic barriers has been attributed to the production of Fas ligand (*120*). The authors based this conclusion on findings that sertoli cells isolated from mice which lack Fas ligand expression (gld) were rejected as opposed to sertoli cells from 'normal' mice which were protected and shown to express Fas ligand (*120*). Concurrently, another group reported that immune privilege of the anterior chamber and cornea of the eye was lost in animal models lacking Fas ligand production (gld), and that 'normal' tissue of the iris, cornea, ciliary body and retina all stained positive for Fas ligand (*121*). Taken together, these results suggest that Fas ligand produced by a tissue can protect it from damaging immune processes, presumably by causing apoptosis of Fas receptor-expressing cells which come into the area.

More recently, murine primary myoblasts were transfected to express FasL and were co-transplanted with allogeneic islets (*52*). Long-term allograft survival was achieved when the islets were accompanied by large numbers of the FasL-expressing myoblasts.

The localized production of the protein encoded by the transfected gene is an important consideration within the Fas/Fas ligand system since, in contrast to CTLA4-Ig, systemic administration of soluble Fas ligand and monoclonal antibodies to Fas receptor produced systemic toxicity in the form of fulminant hepatic failure in mice (*134*).

Neutralization of effector cytokines

Rather than destroying activated cells, another approach would be to neutralize their secreted products. For example, soluble forms of a number of cell surface receptors could theoretically be produced to bind such cytokines as IL-2 and TNF and thereby block immune activation/expansion and prevent the detrimental effects of these cytokines on target islet cells. This approach will not be addressed in these studies.

3. Reprogramming of the overall immune response through production of immune-modulating cytokines.

Another potential approach to prevent graft rejection is to genetically engineer islets to locally express immune-modulating cytokines. The in vitro effects of the 'T_H2-associated' cytokines IL-4, TGF-ß, and IL-10 suggest that some or all of these might be used to inhibit the generation and/or activation of allospecific T effector cells, as well as the production of CTL-associated cytokines (*135*). There have not been any complete published reports of gene therapy trials using either transfected islets or co-transplantable cells which are expressing cytokines. However, one group has presented preliminary results suggesting that adenovirally-transfected islets producing IL-10 and TGF-ß have prolonged survival (*136*). Conversely, work done in Dr. J.F. Elliott's laboratory in Edmonton suggests that IL-10 or IL-4 alone offer no protection (unpublished results). Most of the studies to date have been done using transgenic animals whose islets produce the specific cytokine by virtue of being driven by an insulin promoter (*137-140*). Results have not been encouraging. A significant problem with cytokines is that the cellular response to them is concentration dependent. Therefore, different effects can be obtained depending on the local concentration of the cytokine around the islets. Generally the introduced gene is overexpressed and the cytokine product is therefore present in relatively large amounts.

PURPOSES AND GENERAL OUTLINE OF THESE STUDIES

As previously mentioned, transplantation of insulin-producing tissue is the most physiologic approach to the treatment of diabetes. In order for the use of islet transplantation to become widespread, we must ideally be able to perform it in the absence of systemic immunosuppression. The overall purpose of these studies was to determine whether this could be achieved by transfection of islets with DNA encoding immunomodulating molecules. Localized production of these molecules by the islets themselves, limited to the area of the graft, would theoretically protect the engrafted tissue from immune attack while leaving the rest of the immune system unaffected.

As the University of Alberta is a well-known centre for clinical islet transplantation, the ultimate aim is to use this approach in human islet transplantation. While a mouse model was used to test our hypotheses (being a convenient source of islets as well as a consistent allogeneic combination), it is anticipated that this methodology will be readily transferable to the human islet transplantation setting. With the ultimate goal of human islet transplantation in mind, it was felt that a non-viral transfection technique on intact islets would be most desirable, and that human versions of the immunomodulatory molecules would be cloned and tested.

The first task then, was to determine which transfection technique would be most advantageous and efficacious on intact islets (Chapter II). Having determined that biolistic transfection of islets was possible, a choice of CTLA4-Ig was made as a good candidate for molecular manipulation of the immune response in an in vivo transplant study (Chapter

III). Based on the outcome of our in vivo study, it was felt that the efficiency of the biolistic transfection was sub-optimal, so the biolistic process was studied in more detail (Chapter IV). Finally, two candidate molecules, CTLA4-Ig and soluble Fas ligand, were studied in the transplant setting using the optimized transfection conditions (Chapter V). The final chapter (Chapter VI) includes a general discussion of the results obtained and possible future directions suggested by this work.

Table 1-1. Immunomodulation protocols which have achieved prolonged islet graft survival.

Allografts - mice

1. 12d culture of thyroid tissue at 37°C in 95% O_2:5% CO_2 (*12*).	1975
2. 7-12d culture of islet clusters at 37°C in 95% O_2:5% CO_2 (*141*).	1980
3. Incubation of islets with anti-Ia antibodies and complement (C') (*142*).	1981
4. Treatment of islets with anti-dendritic antibody and C' (*143*).	1985
5. Exposure of islets to gamma radiation (*144*).	1989

Allografts - rats

1. 7d culture at 24°C in 95% air:5% CO_2 and one injection of ALS (*15*).	1979
2. Exposure to ultraviolet radiation (*145*).	1984
3. Incubation with Ia antiserum and C' and 3d of Cyclosporine A (*16*).	1986
4. 7d culture at 24°C and 3d of Cyclosporine A (*16*).	1986
5. Incubation with anti-Ia immunotoxin (*146*).*	1986
6. Exposure to gamma radiation and short course of Cyclosporine A (*17*).	1989
7. 48h culture at 37°C with deoxyguanosine and Cyclosporine A (*18*).	1991
8. 12d culture at 24°C in 95% air:5% CO_2 (*147*).#	1986

* Showed abrogation of stimulating capacity in mixed lymphocyte islet co-culture; no graft performed.
# Showed prevention of autoimmune insulitis.

Allografts - dogs

1. Exposure to ultraviolet-B radiation and low dose Cyclosporine A (*19*).	1990
2. 7d culture at 22°C and low dose Cyclosporine A (*20*).	1991

Xenografts - rat islets to mouse recipient

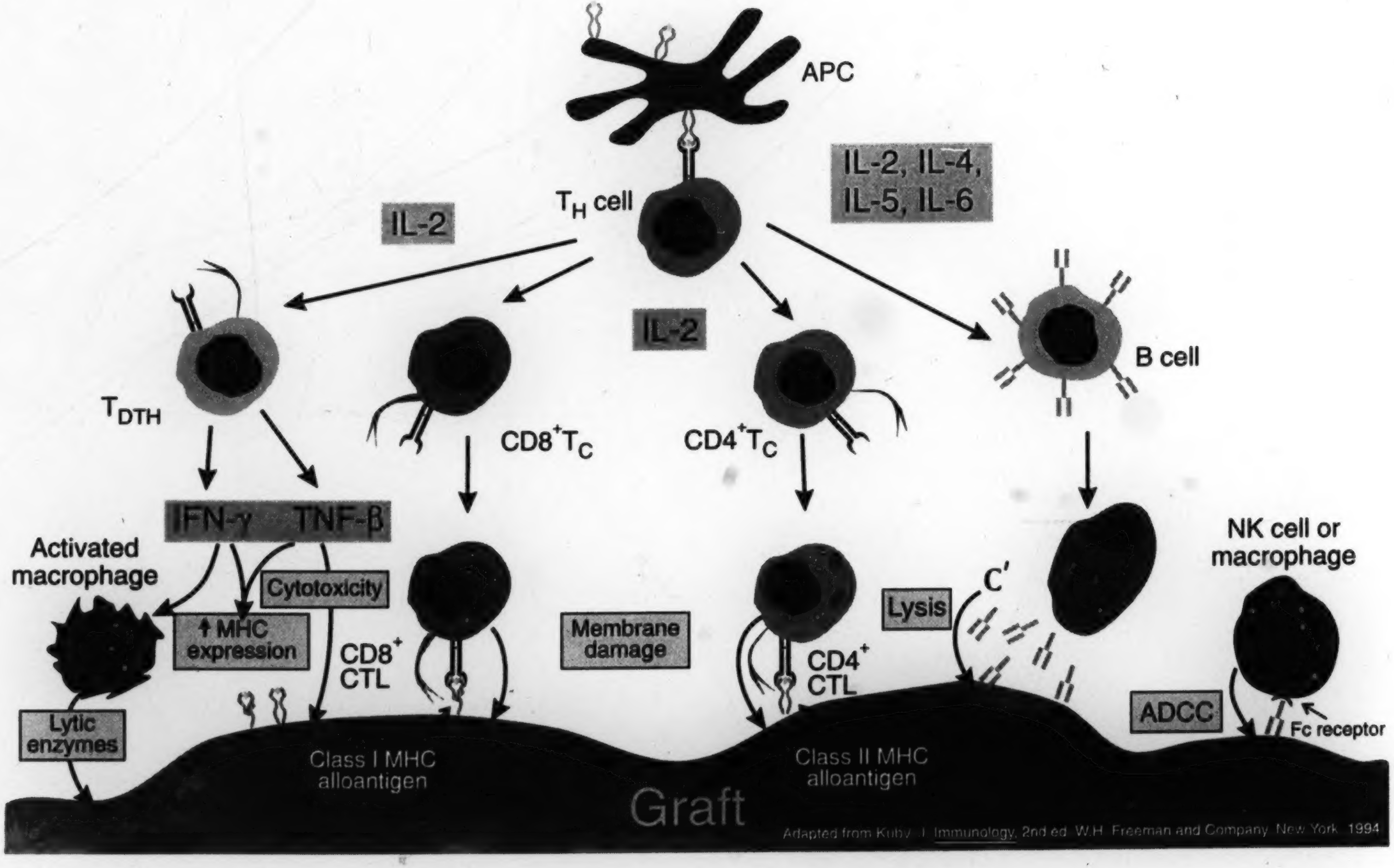
1. 7d culture of mega-islets at 37°C in 95% O_2:5% CO_2 (*148*).	1982
2. Cryopreservation and ALS (*149*).	1987

Xenografts - human islets to mouse recipient

1. 7d culture at 24°C and L3T4 (*21*).	1987
2. Incubation with HLA-I $F(ab)_2$ fragments (22).	1991
3. Injection with B7 blocking agent (CTLA4 Ig) (*23*).	1992

Table 1-2. Summary of transfection methods used with pancreatic islet tissue.

Tissue Type	Method	Species	First Author	Reference
Dispersed Islet Cells	Lipofection	Rat	Welsh, M	(*41*)
		Mouse, rat, pig, human	Saldeen, J	(*39*)
	Electroporation	Rat	Welsh, M	(*41*)
		Fetal rat, ob/ob mouse	Welsh, M	(*40*)
		Rat	German, MS	(*43*)
		Fetal rat	German, MS	(*42*)
	Adenovirus-polylysine DNA	Mouse, rat, pig, human	Saldeen, J	(*39*)
	CaPO4 ppt.	Mouse, rat, pig, human	Saldeen, J	(*39*)
Intact Islets	Lipofection	Mouse	Welsh, M	(*44*)
		Mouse	Gainer, AL	(*45*)
	Adenovirus-polylysine DNA	Mouse, rat, pig, human	Saldeen, J	(*39*)
	Biolistics	Mouse	Gainer, AL	(*45*)
	Adenovirus	Rat	Becker, TC	(*150*)
		Newborn mouse	Csete, ME	(*47*)
		Mouse	Korbutt, GS	(*48*)
		Pig	Mirenda, V	(*49*)
	Herpes virus	Mouse	Liu, Y	(*50*)
Co-transplantation	CaPO4	Mouse/myoblasts	Chahine, AA	(*51*)
	Lipofection	Mouse/myoblasts	Lau, HT	(*52*)


APC
T_H cell
IL-2
IL-2, IL-4, IL-5, IL-6
IL-2
T_{DTH}
$CD8^+T_C$
$CD4^+T_C$
B cell
IFN-γ
TNF-β
Activated macrophage
Cytotoxicity
↑ MHC expression
$CD8^+$ CTL
Membrane damage
$CD4^+$ CTL
Lysis
C′
NK cell or macrophage
ADCC
Fc receptor
Lytic enzymes
Class I MHC alloantigen
Class II MHC alloantigen
Graft

Adapted from Kuby, J. Immunology, 2nd ed. W.H. Freeman and Company, New York, 1994

Figure 1-1. Mechanisms of allograft rejection.

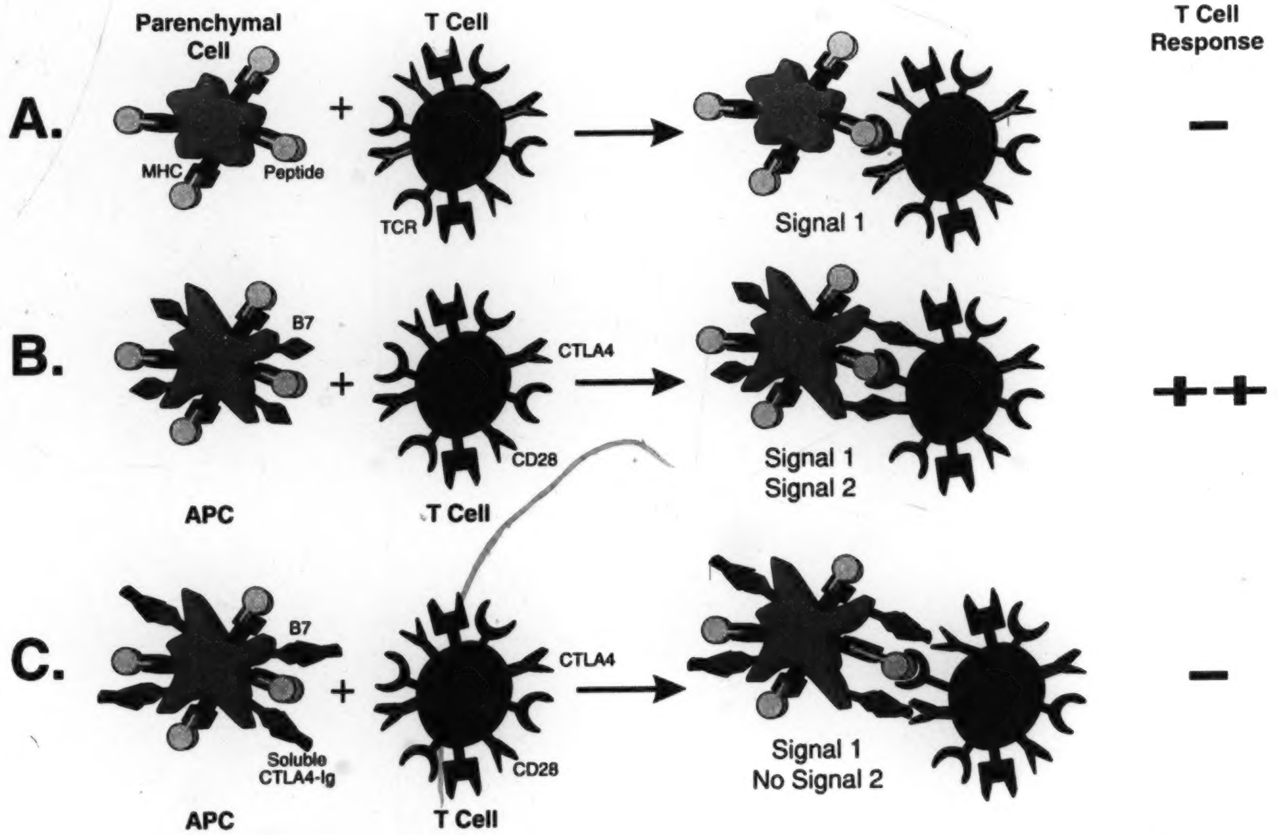

Parenchymal Cell
T Cell
T Cell Response
A.
MHC
Peptide
+
TCR
Signal 1
–
B.
B7
+
CTLA4
CD28
APC
T Cell
Signal 1
Signal 2
++
C.
B7
Soluble CTLA4-Ig
+
CTLA4
CD28
APC
T Cell
Signal 1
No Signal 2
–

Figure 1-2. **The Two Signal Hypothesis of T cell activation.** **A.** Parenchymal cells do not express costimulatory molecules responsible for Signal 2 (eg. B7) so there is no T cell response. **B.** APC expressing B7 leads to T cell response due to presence of both Signal 1 & 2. **C.** Binding of soluble form of CTLA4 receptor (CTLA4-Ig) to B7 on APC prevents interaction with T cell CD28/CTLA4

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CHAPTER II

SUCCESSFUL BIOLISTIC TRANSFORMATION OF MOUSE PANCREATIC ISLETS WHILE PRESERVING CELLULAR FUNCTION[1]

INTRODUCTION

We are interested in using gene therapy to achieve immunomodulation of isolated, intact islets. In these studies, we sought an efficient method to transfect intact islets, which may be practical for application in transplantation.

Biolistic transfection is accomplished by accelerating gold or tungsten particles coated with DNA to high velocity by means of high pressure helium gas. The particles bombard the cells, penetrate their cellular membrane, and enter the cytosol where the exogenous DNA is solubilized and subsequently expressed. The cellular membrane defects undergo repair and the particles themselves are thought to be inert.

Biolistic particle bombardment was first developed for use in plants (*1*). It has since been employed successfully in microbes (*2*), cellular organelles such as chloroplasts (*3*) and mitochondria (*4*), and tissues of live mice (*5*). Recent studies have extended its use to murine cell line systems (*6*) and to *Xenopus* oocytes (*7*). These studies all report high efficiencies of gene introduction, some into primary cell systems which were previously found difficult to transfect, but the effect of the treatment on other cellular functions has not been previously evaluated. We found the biolistic method of transfection of freshly-isolated mouse islets with a reporter gene, firefly luciferase, to be far superior to lipofection using lipofectamine, although inferior to adenoviral infection in terms of transfection efficiency. Furthermore, the in vitro and in vivo function of the islets is preserved.

[1] A version of this chapter has been published in Transplantation 1996, 61: 1567

MATERIALS AND METHODS

1. Islet isolation.

BALB/c mice were anaesthetized using tribromoethanol (Avertin). Pancreatic islets were isolated using collagenase digestion (*8*), and purified on a dextran gradient followed by hand-picking. Islets were isolated and then divided into control and test groups.

2. Molecular constructs.

The firefly luciferase gene driven by the human CMV Immediate Early promoter/enhancer in a pBR322-derived plasmid (kindly supplied by Dr. D. Drucker, University of Toronto) was used as a reporter construct in the biolistic and lipofection transfection efficiency studies. A recombinant E1 deletant of adenovirus 5 containing the firefly luciferase gene under control of the CMV Immediate Early promoter/enhancer (AdCA18-3) was a gift from Dr. Frank Graham of McMaster University, Hamilton. The ß-galactosidase gene driven by the human CMV Immediate Early promoter/enhancer in a pBR322-derived plasmid (pAC CMV ß-Gal, kindly supplied by Dr. R.D. Gerrard, University of Texas, Southwestern Medical Center, Dallas) was used to determine the percentage of transfected cells.

3. Transfection methods.

Gold particles 2.5 μm in diameter (BioRad Laboratories, Hercules, CA) were used as microprojectiles and were found to have good uniformity by scanning electron microscopy (Hitachi S-2500). Particles (30 mg) were washed initially with 95% ethanol, twice with sterile water, then pelleted and resuspended in 500 μL of sterile water (final concentration of 60 mg/mL). DNA precipitation onto the particles was achieved by adding 20 μg (14.3 μL) of DNA (1.4 μg/μL) to 50 μL of Au particles (60 mg/mL), followed by 64.3 μL of 2.5 M $CaCl_2$ (i.e. a volume equal to the volume of DNA and Au particles) and

12.9 μL of 1 M spermidine (1/5 of the volume of $CaCl_2$ used). The mixture was vortexed, left to settle at room temperature for a minimum of 10 min, and spun briefly. The pellet was washed with 70% ethanol, re-pelleted, washed with 95% ethanol, re-pelleted and resuspended in 50 μL of 95% ethanol. The central area of a macrocarrier disc (~7.5 mm in diameter) was then covered with 6 μL of particle slurry (~3 μg DNA) and allowed to dry in a desiccator. For exposure to the biolistic blast, the islets were suspended in 0.10 mL of media on the top of an inverted cell strainer with a mesh size of 40 μm (Falcon). The strainer was encased in a small vacuum chamber, (a modification to a hand-held version of the BioRad biolistic apparatus) resulting in a distance of 25 mm between the stopping screen and the suspended cells (*9*). Particles continued through the screen and bombarded the islets. Initially, groups of islets ranging from 250 to 1350 were used, and the macrocarrier-particle disc was accelerated toward the stopping screen using a helium pressure of either 1000 or 1200 psi under a vacuum of 15" Hg. All subsequent experiments used 1000 islets per exposure to 1200 psi of helium. The islets were resuspended in Ham's F10 media supplemented with 14.3 mM $NaHCO_3$, 10 mM glucose, 2 mM L-glutamine, 10 mM nicotinamide, 0.5 % BSA, 50 mM IBMX (isobutyl-methyl-xanthine), 100 U/mL penicillin and 100 μg/mL streptomycin, and incubated at 37°C in 95% air/5% CO_2. Control groups were put directly into culture.

Lipofection using Lipofectamine (GIBCO/BRL, Burlington, Ontario, Canada) was performed generally according to the manufacturer's instructions. In keeping with their guidelines, we looked at DNA quantities ranging from 0.5 to 3 μg, Lipofectamine amounts from 2 to 8 μL, and islet numbers from 200 to 1300 per mL of final reaction. Optimal efficiency was achieved when 2 μg DNA in 100 μL of serum-free DMEM was added to 6 μL of Lipofectamine pre-mixed in 100 μL of serum-free DMEM, and the mixture incubated at room temperature for 30 min. The DNA:Lipofectamine complexes were then gently added to 1000 islets suspended in 0.8 mL of serum-free DMEM, with frequent mixing. Following 6 h of incubation at 37°C in 95% air/5% CO_2, 4 mL of DMEM containing 10% fetal calf serum was added to the cells and incubation continued for 48 h. Control islets were exposed to the same conditions in the absence of DNA.

Viral infection was performed by adding 0.5 mL of adenoviral stock ($2.5x10^6$ pfu/mL in Ham's F10 containing 2% FCS) to 500 drained, intact islets, and incubating at 37°C for 1 h. The islets were harvested, washed 3 times with Ham's F10, then transferred to 10 mL of Ham's F10 and further incubated at 37°C in 95% air/5% CO_2 for 48 h.

4. Electron microscopy studies.

To demonstrate particle penetration, islets were prepared for conventional scanning electron microscopy (SEM) in standard fashion, immediately following biolistic transformation. This included glutaraldehyde/OsO_4 fixation, ethanol dehydration, critical point drying and mounting. Specimens were sputter-coated with gold and examined (Hitachi S-2500). In addition, islets were cultured for 24 h and then subjected to natural SEM. In this case, samples were prepared initially as outlined above, but were not sputter-coated with gold. They were viewed directly using a natural scanning electron microscope (Hitachi S-2460N). An electron accelerating voltage of 5 kV was used initially, followed by an electron accelerating voltage of 15 kV so that the electron beam penetrated to a deeper level within the cells.

5. Characterization of biolistically transfected islets.

Recovery of intact islets following exposure to the biolistic blast was determined by recounting the exposed islets following 48 h of culture and expressing the results as a percentage of the original. To determine the percentage of remaining cells expressing the foreign gene following biolistic transfection, islets that had been blasted with the ß-galactosidase gene were harvested following 48 h of culture and dissociated into single cells in a medium of 123 mM NaCl, 5.4 mM KCl, 0.4 mM KH_2PO_4, 1.0 mM Na_2HPO_4, 4.2 mM $NaHCO_3$, 0.8 mM $MgSO_4$, 10 mM HEPES, 1 mM EGTA and 2.8 mM D-glucose

(*10*). The cells were fixed for 5 min at room temperature in a solution of 1 % formaldehyde and 0.2 % glutaraldehyde in PBS. Following thorough washing in PBS, the cells were immersed in 0.5 mL of stain composed of 4 mM potassium ferrocyanide, 2 mM $MgCl_2$, 4 mM potassium ferricyanide and 0.4 mg/mL of X-galactosidase (5-bromo-4-chloro-3-indolyl-b-D-galactoside) and incubated at 37°C for 12-16 h. The percentage of cells expressing the ß-galactosidase gene was determined by manually counting the proportion of cells that appeared bright blue in colour under light microscopy.

6. Luciferase assays.

To determine transfection efficiency, the groups of 1000 islets (or 500 in the case of the viral transfections) were harvested after 48 h of culture and washed 3 times with fresh media. The resulting pellet was mixed with 50 µL of cell lysis buffer consisting of 0.1 M Tris pH 7.8, 0.5% NP 40, and 1.0 mM dithiothreitol and the lysate spun in a microcentrifuge x 16,000 g for 15 min at 4°C. Luciferase enzyme activity was measured immediately on a Lumat LB 9501 (Berthold) using 20 µL of cellular lysate in 100 µL of assay buffer consisting of 0.5 mM luciferin in 20 mM Tricine, 1 mM $MgCO_3$, 2.7 mM $MgSO_4$, 0.1 mM EDTA, 0.03 M DTT, 0.5 mM ATP, 0.3 mM Coenzyme A, and 1 ng/mL bovine serum albumin (Sigma Fraction V), final pH 7.8. Results are expressed as relative light units (RLU)/islet.

To determine the kinetics of gene expression over time, islets were biolistically transfected with the gene for firefly luciferase and cultured for 24 h at 37°C. Hand-picked islets (500) were transplanted under the kidney capsule of diabetic mice as described below. Nephrectomies of the engrafted kidneys were performed over various time intervals, and the organs either snap frozen in liquid nitrogen or extracted for luciferase immediately. Extraction was achieved by initially forcing the kidney through a fine metal mesh and then rinsing with 150 µL of cold PBS. To this, 150 µL of cold 2X lysis buffer (see above) was added. Following thorough mixing, samples were spun in a microcentrifuge at 4°C for 15 min and assayed as above.

7. Functional studies.

Following 24 h of culture, in vitro islet function (50 islets/assay) was assessed by determining insulin release after a 2 h static glucose challenge using either 2.8 mM or 20.0 mM glucose. Stimulation indices were calculated by dividing the insulin release at 20.0 mM glucose by the insulin release at 2.8 mM glucose. In vivo function after 24 h of culture was determined by the ability of 500 islets to reverse the diabetic state when transplanted under the kidney capsule of a syngeneic, diabetic recipient, anaesthetized with halothane. Diabetes was chemically-induced using 90 mg/kg of alloxan via the penile vein. Glycemic response was determined by assay of capillary blood glucose using a Medisense Companion 2 glucometer following tail snips. Reversal of diabetes due to graft function was confirmed in all cases by return to the diabetic state following nephrectomy of the engrafted kidney.

RESULTS

1. Optimization of the biolistic procedure.

Figure 2-1 shows the luciferase activity obtained from a range of different islet numbers per blast using He pressures of 1000 and 1200 psi to accelerate the gold particles. Gene expression was consistently higher using the higher helium pressure. Expression also increased as islet number per exposure increased up to a level of 1000 islets. Beyond this, expression dropped off at 1000 psi probably due to a larger number of islets not being penetrated by particles. At helium levels of 1200 psi, only a small increase in expression was achieved above 1000 islets. Therefore, due to limitations in islet availability, parameters of 1200 psi of helium and groups of 1000 islets were chosen for subsequent experiments.

2. Particle penetration.

Figure 2-2 is a conventional scanning electron micrograph of a BALB/c mouse pancreatic islet taken immediately after biolistic exposure. A hole in the cellular membrane, presumably caused by particle entry since it is of compatible size, is clearly visible. Figure 2-3 shows electron micrographs using natural SEM. When an electron accelerating voltage of 5 kV was used, a few gold particles are seen on the islet surface as highly refractile spheres (Fig 2-3A). With an increase in the electron accelerating voltage to 15 kV, the electrons in the beam travel a further distance into the sample, and many more particles are visible (Fig 2-3B), indicating at least some degree of penetration of the particles into the islet.

3. Recovery and efficacy of transfection.

The mean recovery of islets following exposure to the biolistic blast (n=20) was 53%; in other words, 47% of the islets are lost simply through exposure to the process. Of the remaining blasted islets, an average of 3% of dissociated cells stained positively for expression of the introduced b-galactosidase gene (n=6). Data for islets from 4 separate islet isolations, subjected to independent transformations by either biolistics, lipofection or adenoviral infection is presented in Table 2-1. The biolistic method achieved, on average, a 35-fold higher level of luciferase activity than did lipofection using Lipofectamine. Adenoviral infection achieved, on average, a further 25-fold increase over that of biolistics. There is considerably more variability within the biolistic and adenoviral groups than the lipofectamine group (mean±SEM of 42.6±14.2 and 1136.0±271.0 RLU/islet compared to 1.1±0.2 RLU/islet respectively). Luciferase activity was not detected in control islets.

Extraction of biolistically-transformed islet grafts (500 islets/graft) and assay for luciferase activity indicated a transient in vivo expression, with levels of 24,070 RLU and 26,233 RLU at 48 h and 1 wk post-transfection, respectively, dropping to 9,747 RLU and 3,155 RLU by 3 and 4 wks, respectively.

4. Functional studies.

Table 2-2 demonstrates in vitro insulin release by biolistically-transfected islets after glucose challenge. The mean±SEM stimulation index was 2.8±0.3 in the non-transfected, control group versus 3.0±1.0 in the biolistics group. Although there were trends to increased insulin release by the biolistically exposed islets, there was no significant difference between the groups in terms of their ability to respond to an in vitro glucose challenge ($p=0.9$). Figure 2-3 shows the function of biolistically-transfected islets in vivo. Although 2 cases took longer to reach completely normal blood glucose levels, hyperglycemia was reversed in 7 of 7 mice that received biolistically-transfected islets. Prompt return to hyperglycemia was seen after nephrectomy of the graft-bearing kidneys, conclusively proving dependence on islet graft function for diabetes reversal.

DISCUSSION

When considering transplantation of insulin-producing tissue for the treatment of diabetes, it has been shown previously that the function of isolated beta cells is compromised, compared to beta cells in the presence of the other endocrine cells normally present in an intact islet (*11*). To pursue gene therapy approaches, we therefore desired a method to achieve transfection of intact islets, since we hope to achieve long-term reversal of the diabetic state. Previous transfection studies using either lipofection (*12*) or electroporation (*13*) attained modest levels of transient gene expression when monolayers of cells prepared from dispersed islets were used. However, one would not expect that these techniques would achieve the same efficiency on intact islets, which are a cluster of some 2,000 cells surrounded by a network of collagen and connective tissue. In spite of this, successful transfection of intact islets using lipofectin has been reported (*14*). Viral vectors would be expected to be capable of penetrating the cellular clusters, although, since an islet is composed mainly of non-dividing cells, existing retroviral vectors cannot be employed. Adenoviral vectors are being used successfully in several eukaryotic systems including islets (*15-18*), but immune responses to the carrier virus have been documented (*19*). As we are working within a transplantation framework, we desired a method of transfection which would leave the transfected cells as immunologically unaffected as possible. Transfection by particle bombardment would theoretically overcome many of the above problems. Since the particles are accelerated to high velocity, they are capable of penetrating several cell layers, including connective tissues (*5*), so cellular access should not present a major problem. Additionally, there should be no immunological response since there is no introduction of viral proteins or viral gene products to the system. There is no requirement for actively dividing cells and the method has been used successfully in several types of primary cells.

Our natural scanning electron microscopy studies indicate that the gold particles are not simply situated on the surface of the islet, but have penetrated the islet to at least some degree. We attempted to use transmission electron microscopy to detect individual cells containing gold particles, but with so few cells affected, it was an extremely time-consuming and costly task and had to be abandoned. However, there is no doubt that at least some of the particles are indeed penetrating the cells, since in order to detect any luciferase activity at all, the DNA has had to have been internalized, solubilized, transcribed and translated into a functional protein. These processes all occur intra-cellularly. It is possible that the particles are predominately penetrating the islet periphery, that is, the glucagon-secreting alpha cells. Depending on the ultimate aim to be achieved, however, it may in fact be beneficial for a non-beta cell to be producing the foreign protein thus leaving the beta cells unaffected.

We have demonstrated that after dissociation of islets into single cells, the proportion of the transformed cells producing the foreign gene product is small (i.e. about 3%). It is possible that some of the peripheral cells are damaged and lost during the dissociation process, making this experimental value somewhat lower than the real value. Although the exact gene copy number per transformed cell is difficult to determine in all the methods of transfection used in this study, we do know that each particle carries in many molecules of DNA. For this reason, although we have included data to show the apparent percentage of cells transfected, we feel that a more meaningful comparison of expression efficiency is obtained using the total levels of reporter protein production per islet. However, total levels of luciferase obtained by biolistic transformation versus recombinant adenoviral infection (roughly 25-fold higher for the adenovirus), is in keeping with the proportion of cells transfected per islet by these two different methods (~3% for biolistics versus ~50% by adenoviral infection at the viral titres used; data not shown).

Transfection of intact islets by the biolistic method does, however, present its own unique problems. Since islets are aggregates of cells which remain in suspension, we had to develop a novel system to allow exposure of the islets to the blast, since all previous in vitro biolistic studies involved eukaryotic cells which were attached to a culture dish. The system we developed does result in some variability in the number of cells which contain particles, and a great number of particles do not actually collide with a cell but are seen merely suspended within the media.

Using our system, there was a significant degree of immediate islet damage seen following the biolistic blast. This was not unexpected considering the nature of the process, and seemed to be the result of exposure to both the high pressure helium shock wave, and the high level of vacuum. The damage manifested as dispersal of approximately 50% of the islets into free floating cells in the media and an obvious loosening of the cells around the perimeter of the remaining islets Following the culture period, surviving islets appeared to regain a more compact appearance. Despite the loss, however, whole islets which did survive the treatment appeared to have similar in vitro and in vivo function compared with untreated controls, and also clearly continued to express the reporter gene product both in vitro and in vivo. With such a small number of cells being targeted by the particles, it may not be the transfected cells themselves which are responsible for insulin production, although this remains to be proven. We have shown, however, that islets exposed to both the vacuum and shock wave were still capable of normal function.

We feel this methodology offers an alternative for efficient transfection of intact islets, since cellular function is preserved and immunological status should not be adversely affected. It therefore holds promise for ex vivo gene therapy of isolated islets where genes for immunomodulating molecules such as cytokines, adhesion molecule-blocking or signal-blocking agents could be introduced into the islets before transplantation, thus rendering them more immunologically acceptable to a recipient.

Table 2-1. Efficacy of gene introduction into mouse islets.

Group (n=4)	Luciferase Activity (RLU/islet)				(Mean±SEM)
Lipofection	1.6	1.4	0.8	0.6	1.1±0.2
Biolistics	64.2	69.3	24.8	12.1	42.6±14.2
Adenovirus	869.8	545.7	1348.8	1776.6	1136.0±271.0

Table 2-2. Insulin response of islets to static glucose challenge following biolistic transformation.

Group (n=4)	Insulin release 2.8 mM glucose (pmol/islet)	Insulin release 20.0 mM glucose (pmol/islet)	Stimulation index
Control	125	353	2.8
	125	359	2.9
	147	449	3.1
	204	471	2.3
Mean±SEM	150±18	408±61	2.8±0.3
Biolistics	138	581	4.2
	146	482	3.3
	248	609	2.5
	413	836	2.0
Mean±SEM	236±64	627±150	3.0±1.0[a]

[a] P=0.9, Mann-Whitney U test

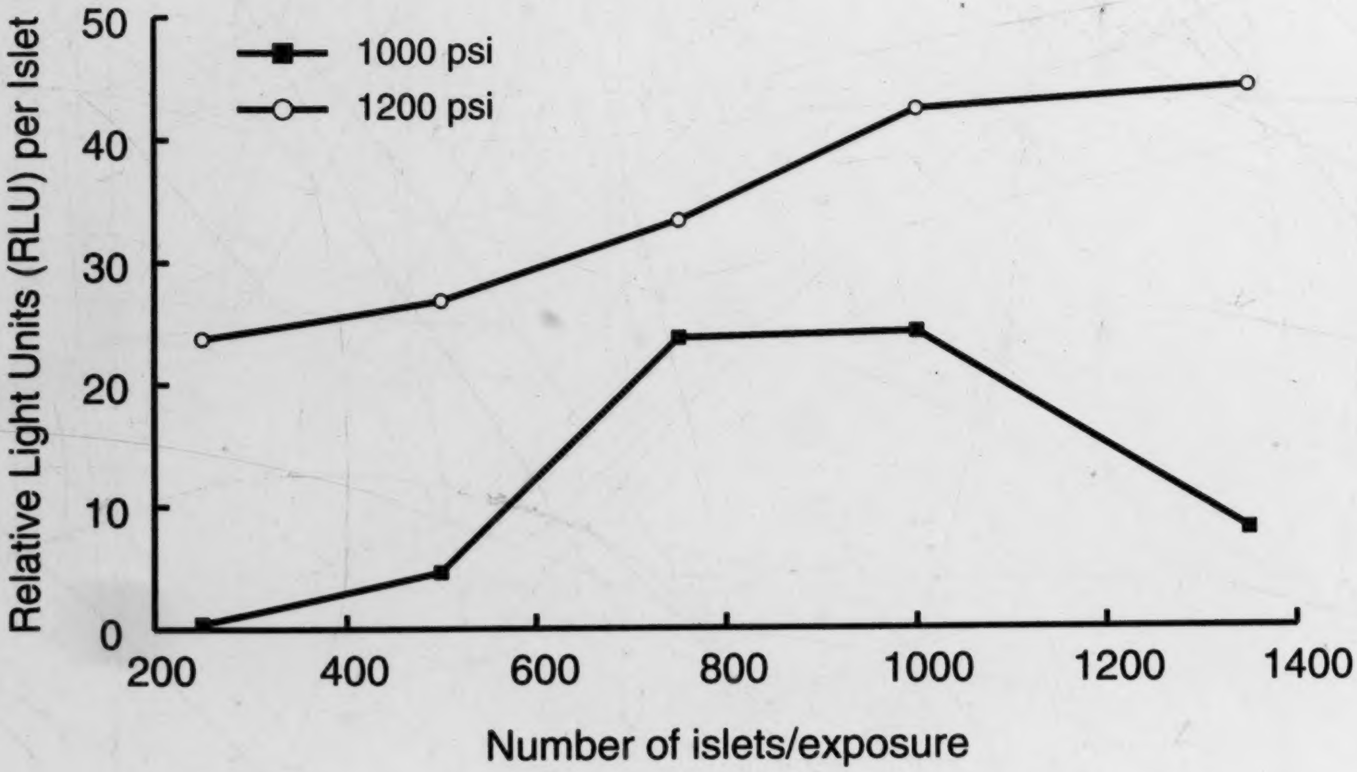

Figure 2-1. Effect of helium pressure and number of islets per biolistic exposure on reporter gene expression as assayed by luciferase production.

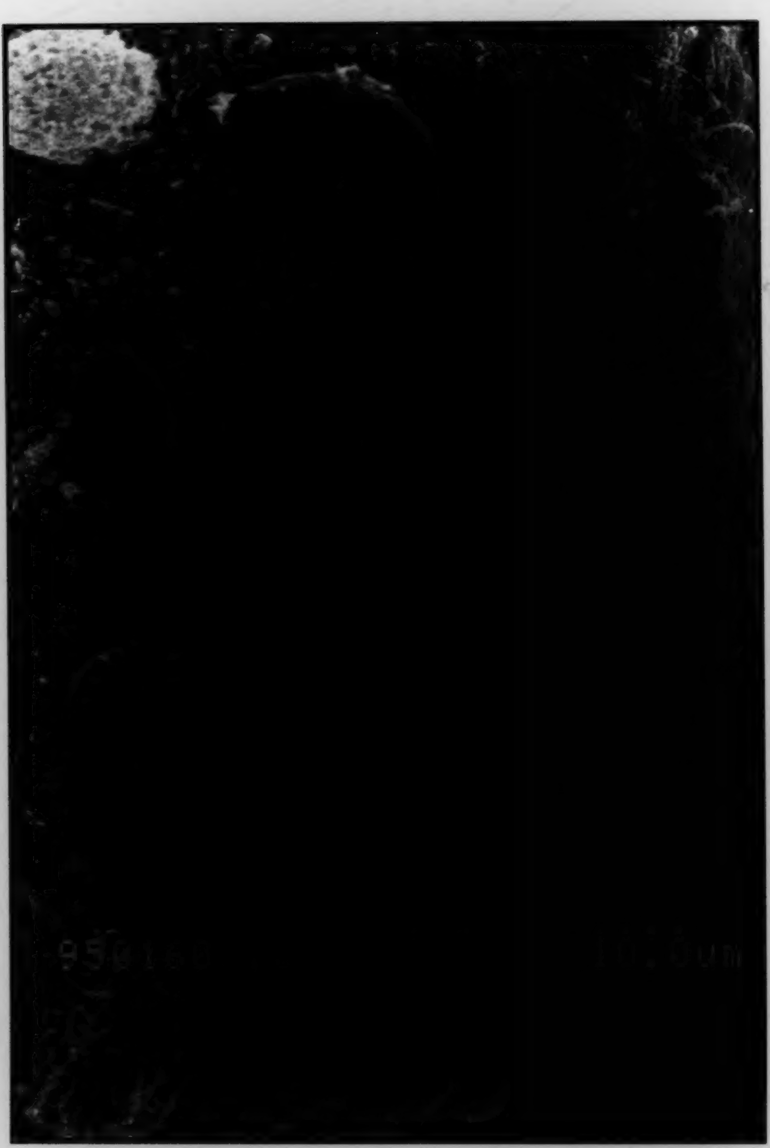

Figure 2-2. Scanning electron micrograph of a BALB/c mouse islet immediately after biolistic transfection, showing a hole 4 µm in diameter in the cellular membrane (particles are approximately 2.5 µm in diameter).

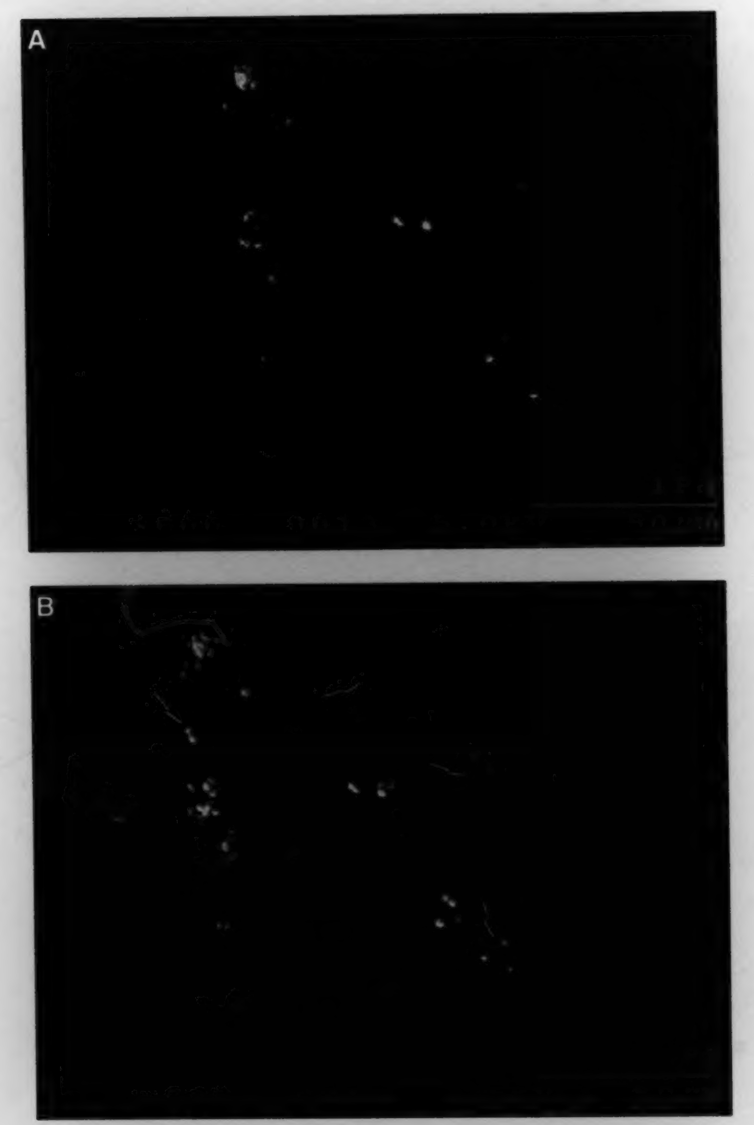

Figure 2-3. Natural scanning electron micrographs of the same biolistically transfected BALB/c mouse islet examined at two different electron-accelerating voltages.

A) Using an electron-accelerating voltage of 5 kV, a small number of gold particles can be seen on the surface of the islet.

B) Using an electron-accelerating voltage of 25 kV, a large number of particles can now be seen (arrows), indicating that the majority of the particles have a deeper intracellular or intercellular placement.

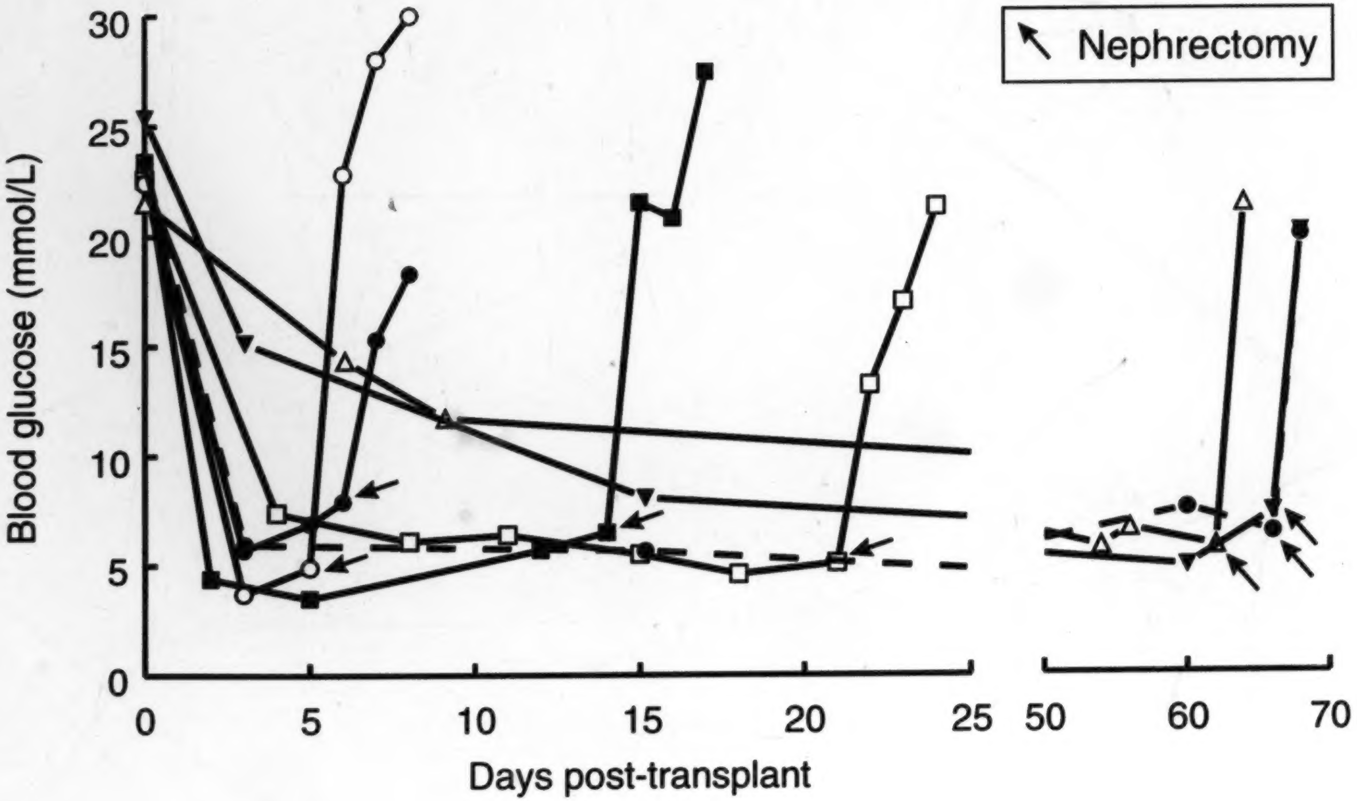

Figure 2-4. *In vivo* function of seven biolistically transfected BALB/c islets transplanted under the kidney capsule (500 islets/mouse) of alloxan-induced diabetic BALB/c recipients. Arrows indicate time of nephrectomy of the graft-bearing kidneys.

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CHAPTER III

EXPRESSION OF CTLA4-IG BY BIOLISTICALLY-TRANSFECTED MOUSE ISLETS PROMOTES ISLET ALLOGRAFT SURVIVAL[1]

INTRODUCTION

Although great advances have been made in organ transplantation, all of the agents commonly used as systemic immunosuppressants have toxic side effects. Localized delivery of immunosuppressive molecules to the site of the engrafted tissue could theoretically ameliorate many of the hazardous effects of these agents on unrelated tissues, as well as avoid systemic immunosuppression which leaves the recipient vulnerable to infectious agents. In islet transplantation, local production of an immunoprotective protein could be achieved by co-transplantation of unrelated tissues which express the desired protein (*1*, *2*). Alternatively, the islets themselves could be transformed to produce the immunoprotective molecule. It is the latter option which we have explored in the present study.

Several investigators have shown prolongation of allograft survival using systemically-administered CTLA4-Ig (*3-5*). Local production of murine CTLA4-Ig by co-transplanted cells derived from a muscle cell line also resulted in prolonged islet allograft survival when the muscle cells were syngeneic to the recipient (*1*). Ex vivo coating of mouse islets with murine CTLA4-Ig protein has also achieved long-term survival in a portion of allografts (*6*). For these reasons, we felt that CTLA4-Ig was a good candidate for an islet transplantation/gene therapy trial.

We have previously established a transfection method for islets using biolistics

[1] A version of this chapter has been accepted for publication. Gainer AL, Korbutt GS, Rajotte RV, Warnock GL, Elliott JF. Transplantation 1997.

which results in transient gene expression (*7*). We now find that introduction of the gene for human CTLA4-Ig into CBA islets using this method results in statistically significant prolongation in allograft survival when the transfected islets are transplanted into diabetic BALB/c recipients.

MATERIALS AND METHODS

1. Islet isolation.

CBA mice (Jackson Laboratories, Bar Harbor ME) were anesthetized using tribromoethanol (Avertin). Pancreatic islets were isolated using collagenase digestion (*8*) and purified on a dextran gradient. The islets were cultured overnight in 95% air/5% CO_2 at 37°C in Ham's F10 media supplemented with 14.3 mM $NaHCO_3$, 10 mM glucose, 2 mM L-glutamine, 10 mM nicotinamide, 0.5% bovine serum albumin, 50 mM IBMX (isobutyl-methyl-xanthine), 100 U/mL penicillin and 100 µg/mL streptomycin.

2. Molecular constructs.

The cDNA encoding human CTLA4-Ig was modeled essentially on the construct of Linsley et al. (*9*). Our construct contained three DNA segments encoding the following elements: 1) the oncostatin M signal sequence (amino acids 1 to 25), 2) the extracellular domain of human CTLA4 (amino acids 26 to 150), and 3) the human Fcγ1 region, with cys to ser substitutions for all cys residues in the hinge region (amino acids 151 to 383). Our cloning strategy and the complete cDNA sequence appears in the Appendix (pages 162, 164). The cDNA encoding the fusion protein was excised with Xba I and Not I and inserted into a pUC-based eukaryotic expression vector (pPSC1) which contains the human

CMV (Towne strain) Immediate Early 1 promoter/enhancer + Intron A, as well as a downstream polyadenylation signal from SV40 virus (J.F.E., unpublished).

3. Transfection methods.

Islet transfection was performed as previously described (*7*). Each macrocarrier disc held 0.5 mg of 2.5 µm gold particles (BioRad, Mississauga, Ont) coated with 3.3 µg of DNA -- either the pPSC1 vector alone, or the same vector containing the CTLA4-Ig cDNA. One disc was used to blast 1000 islets. Initially the rupture disc was pierced manually, with the helium pressure set to 1200 psi, but since we could not be sure of the actual pressure of the shock wave released, we switched to using spontaneous rupture of discs graded to 400 psi. Following transfection, the islets were cultured for a further 40-48 h using the same conditions as described above.

4. Functional studies.

Following the culture period, transfected CBA islets were hand-picked (500 islets/recipient) and placed under the kidney capsule of alloxan-induced diabetic BALB/c recipients (90 mg/kg via the penile vein). Blood glucose was determined using a Medisense Companion 2 glucometer on samples taken from tail cuts. Readings were taken three times per week until the high risk period for rejection had passed (25 days), then once a week, on average. Grafts were deemed to have failed when blood glucose levels rose from initial normoglycemic levels (<10 mmol/L) to exceed 16.5 mmol/L (confirmed by a second reading taken 24 h later).

5. Histologic methods.

Following nephrectomy, engrafted kidneys were placed in Bouin's fixative for 2 h, and then paraffin-embedded. Mounted sections underwent routine hematoxylin/eosin and aldehyde fuchsin staining. Immunohistochemical staining of paraffin sections for insulin was performed at room temperature. The staining involved quenching with 10% hydrogen peroxide/80% methanol for 6 min, blocking for 15 min with 20% horse serum, incubation for 30 min with a 1:1000 dilution of primary guinea pig anti-porcine insulin antibody (DAKO Corp., Carpinteria, CA), and for 20 min with a 1:200 secondary biotinylated goat anti-guinea pig IgG antibody (Vector Laboratories, Burlingame, CA). A Vectastain ABC kit (Vector Laboratories) and a liquid DAB Substrate Pack (BioGenex, San Ramon, CA) were used for color development, per the manufacturers' instructions.

RESULTS

1. Allograft survival.

Following exposure to the biolistic blast, the majority of the CBA islets appeared intact, with a visible capsule. Using an inverted microscope and a magnification of 100X, gold particles could be clearly seen within most of the islets (data not shown).

Table 3-1 shows the survival of the various transfected islet allografts. Functional graft survival for control islets transfected with the empty expression plasmid vector ranged from 8-19 days, (mean ± SD, 12.8 ± 3.6 days), with no grafts surviving beyond 19 days. Amongst the CTLA4-Ig-transfected grafts, 6 of 12 survived similar to the control group, but the other 6 grafts survived until the time of nephrectomy at 50, 130 or 164 days (n=2 each). Overall, survival of the CTLA4-Ig-transfected islets was 66.8 ± 61.5 days (mean ± SD), when the day of nephrectomy was considered as the duration of graft survival. This differed significantly from the control group (p=0.01, Fisher's Exact Test for survival to 50 days). In 4 of the 6 CTLA4-Ig grafts surviving long-term, the animals reverted to the diabetic state following nephrectomy to remove the graft. In the remaining two animals, blood glucose levels remained within the normal range, indicating some return of endogenous pancreatic islet function during the post-transplant period. However, macroscopically both of these kidneys had significant islet tissue present in the graft site at the time of nephrectomy. Further, microscopically both grafts showed abundant well-granulated islet tissue present when histological and immunohistochemical analysis was performed (see below).

Figure 3-1 shows the blood glucose readings for the six BALB/c mice which had long-term survival of CTLA4-Ig-transfected CBA islets. In all cases, the recipient's blood glucose levels returned to < 10.0 mM by the second day post-transplantation. Four of the six recipients had quite stable readings over time, while two cases showed more variability in their blood sugar levels.

2. Histological appearance of grafts.

Figure 3-2 shows the histological and immunohistological appearance of a representative set of grafts, including a rejected control allograft (Fig. 3-2A; islets transfected with the empty plasmid vector alone), as well as CTLA4-Ig-transfected allografts which survived longer term, and were harvested at 50 (Fig. 3-2C), 130 (Figs. 3-2B and 3-2D) and 164 (Figs. 3-2E and 3-2F) days post-transplantation. Although there are still the remnants of a cellular infiltrate in the rejected control graft (Fig. 3-2A), the islets have been destroyed and largely replaced by fibrotic tissue. In longer surviving CTLA4-Ig-transfected grafts, islets staining intensely for insulin were present. This includes the 164 day graft shown in Figs. 3-2E and 3-2F, which is from one of the two animals which did not return to the diabetic state following nephrectomy. In other words, despite having regained some pancreatic function, there was still a very large insulin-producing graft present in the kidney. The long-term grafts are all accompanied by dense accumulations of mononuclear cells, although to variable degrees. Figure 3-2E and 3-2F are tissue sections taken at different levels within the same islet graft, but clearly show a significant difference in the number of mononuclear cells present. In all of the sections from long-term grafts, the mononuclear cells appear in islands adjacent to the islets and there is no lymphocytic infiltration into the grafts (Fig. 3-2B shows detail of the 130 day graft shown in Fig. 3-2D).

DISCUSSION

Localized immunosuppression through gene therapy is attractive in islet transplantation because it has the potential to reduce or eliminate the need for systemic immunosuppression. This approach would provide specificity of immunosuppression, leaving the rest of the immune system unaffected. Stable transfection of the islets would be ideal, but this is not possible with currently available techniques. However, methods which achieve even transient expression can be used to prove the principle and to assess which genes will be most useful. With expression of human CTLA4-Ig, attained through biolistic transfection of mouse islets, we have achieved a long-term islet allograft survival rate of 50 % in a strongly allogeneic strain combination. This compares favorably with the success achieved when murine CTLA4-Ig was locally produced by co-transplanted muscle cells, where survival >51 days was observed in only 1 of 10 grafts (*1*). There are at least two possible explanations for these differing results. First, the close proximity of CTLA4-Ig molecules originating from intra-islet CTLA4-Ig-producing islet cells and B7 molecules on passenger APCs may facilitate blocking more effectively than the peripheral location of CTLA4-Ig-producing myocytes and the same APCs. Second, it was suggested by Chahine and colleagues that the muscle cells probably produce low levels of the CTLA4-Ig protein initially, while recovering from the shock of transplantation. This would not be such a concern in our case, since many of the B7 sites would already be blocked at the time of transplantation due to local protein production and binding during the pre-transplant in vitro culture period. Further limitations of the co-transplantation approach using immortalized cell lines are the possibility of tumor formation and the necessity of the transplantable cell line tissue being syngeneic to the recipient. Lau et al. have more recently co-transplanted transfected primary myoblasts, rather than muscle cells derived from a cell line (*2*). However, this necessitates obtaining syngeneic tissue from the recipient before islet transplantation, which may not be clinically practical.

The fact that the direct pathway of antigen presentation (i.e. donor APCs) plays a major role in allograft rejection was confirmed by the study of Steurer et al. (*6*). When islets were pretreated with murine CTLA4-Ig before transplantation, 42 % of allografts survived long-term. The authors suggested that graft failures may have occurred because B7 expression was induced following transplantation, and there was no further exogenous CTLA4-Ig available for blocking. In essence, we are achieving a similar state of affairs, except that our protein is produced locally by the islets in situ rather than being added exogenously. In our gene therapy approach, the induced B7 should be blocked at least initially, since we anticipate ongoing production of CTLA4-Ig by the islets for at least a period of several weeks.

The failure to achieve higher long-term survival rates in our study may be due to a number of factors. In those grafts which fail, it is most likely that levels of CTLA4-Ig protein are not sufficient to provide complete blockage of all B7 sites. We have previously shown considerable variability in the amount of reporter protein produced by biolistically-transfected islets (*7*), probably for technical reasons inherent to the biolistic process itself, and we are continuing to explore this problem. Additionally, although human CTLA4-Ig has been shown to bind to murine B7 efficiently (*10*), murine CTLA4-Ig binds with a 10-fold higher affinity (*6*), and thus use of the murine gene might be expected to provide more complete B7 blocking and a higher success rate.

Our long-term allografts were accompanied by dense mononuclear cell peri-infiltrates. This phenomenon was also observed by Steurer et al. (*6*) who identified the cells as predominantly CD4+, with a minority of CD8+ cells also present. A similar, if not identical final picture is also obtained when syngeneic islet grafts are transplanted into diabetic non-obese diabetic mice, simultaneous with the injection of complete Freund's adjuvant (*11*). Again, the majority of T cells present were found to be CD4+. In all models, the function of these accompanying mononuclear cells remains an enigma, but some form of dominant bystander immunosuppression must be considered.

In summary, we have shown that local production of CTLA4-Ig by biolistically-transfected islets can provide long-term protection for mouse islet allografts. With continued technical advances in the biolistic transfection procedure, we anticipate that the rate of success would further increase. This model provides all the advantages of supplying specific immunoprotection localized to the site of the graft, without the disadvantages of having to create an appropriate syngeneic accompanying cell for co-transplantation or of having to create a recombinant virus for each and every immunomodulating molecule to be tested. Thus the approach can be used to rapidly screen immunomodulating molecules alone or in various combinations, to determine which molecule(s) will be most useful for blocking rejection responses.

Table 3-1. Survival of islet allografts: BALB/c mice transplanted with 500 biolistically transfected CBA islets.

DNA used for transfection	Number of days of post-transplantation normoglycemia (mean ± SD)
pPSC1 vector only (n=10)	8, 8, 10, 11, 13, 13, 14, 15, 17, 19 (12.8 ± 3.6)
CTLA4-Ig in pPSC1 vector (n=12)	10, 12, 18, 20, 20, 33, 50[a],50[a], 130[a,b], 130[a], 164[a,b], 164[a] (66.8 ± 61.5)[c]

[a] nephrectomy performed at this time

[b] blood glucose failed to return to the diabetic range following nephrectomy, but harvested grafts showed well-granulated islets (see Figs. 2E and 2F for the 164 day graft)

[c] P = 0.01 (Fisher's Exact Test for survival ≥ 50 days)

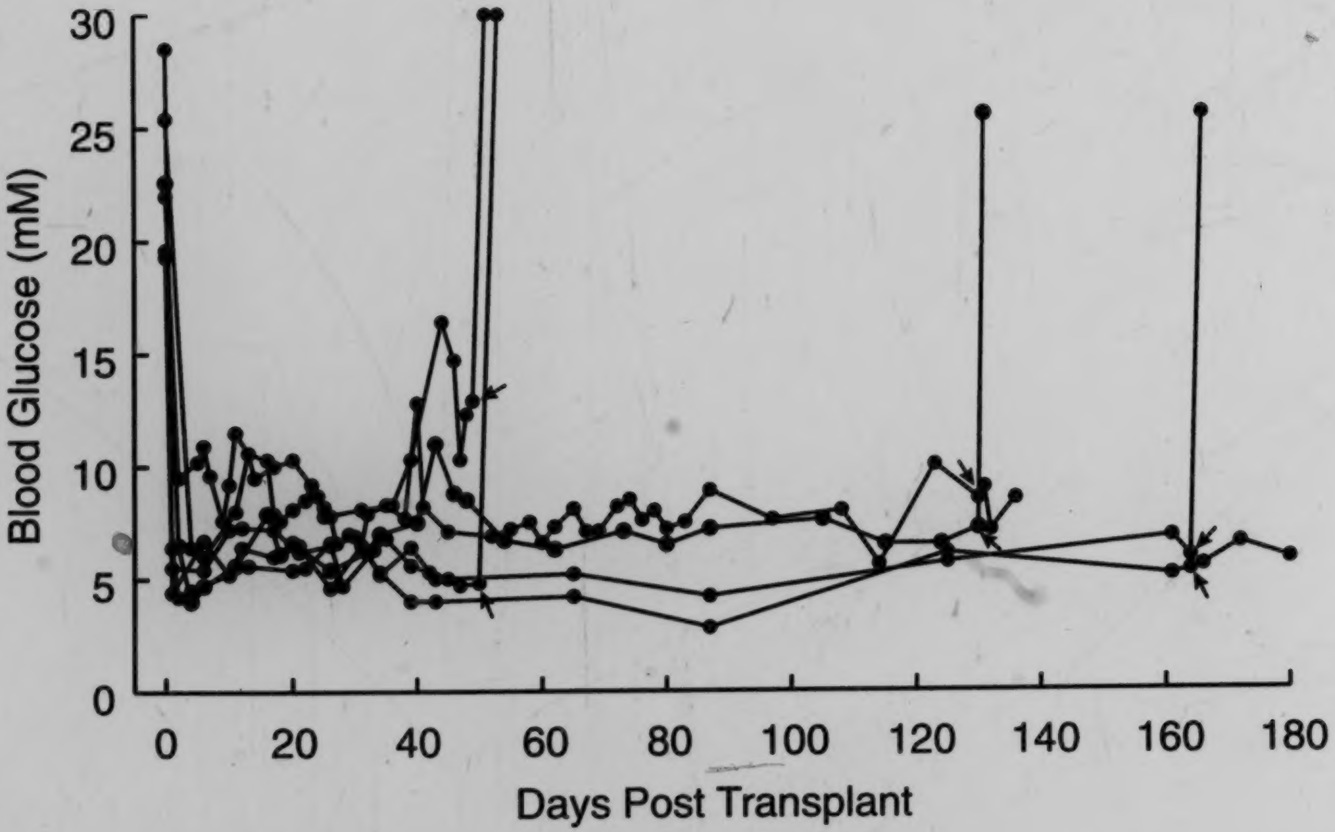

Figure 3-1. Blood glucose concentrations in six alloxan-induced diabetic BALB/c mice transplanted on day 0 with 500 CBA islets which had been biolistically transfected with human CTLA4-Ig. Arrows indicate the time of nephrectomy of the engrafted kidneys.

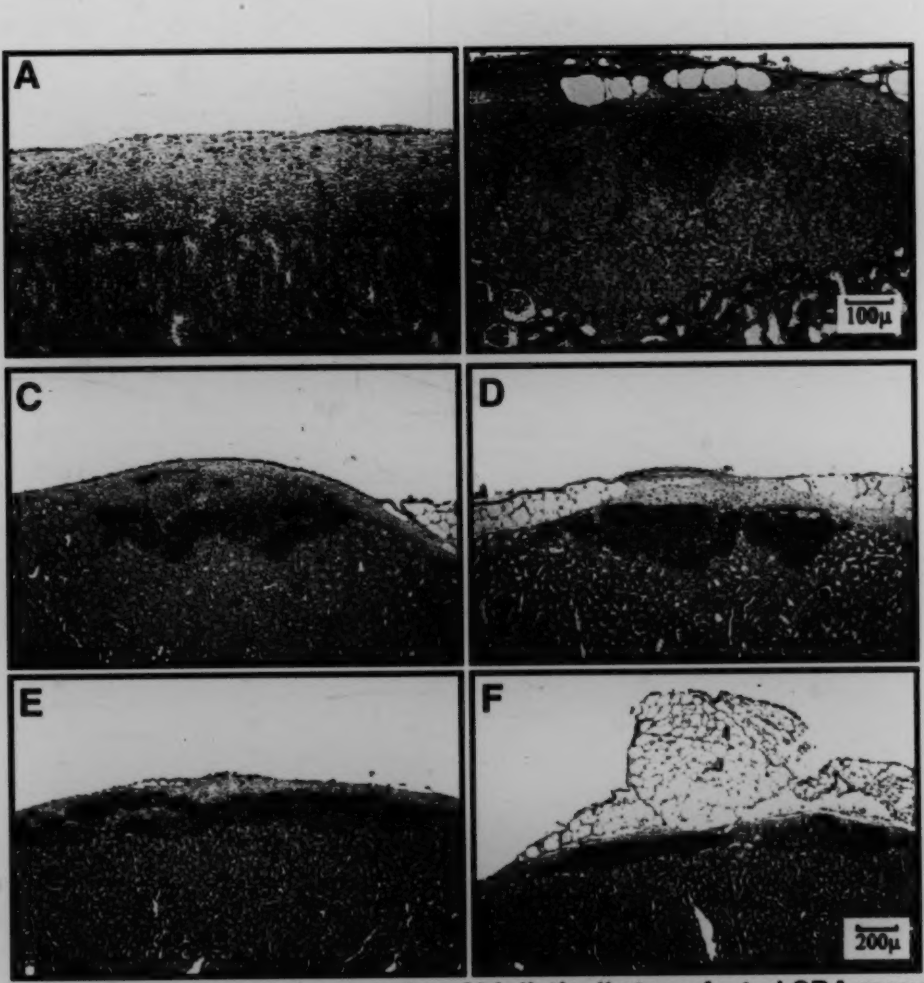

Figure 3-2. **Photomicrographs of biolistically transfected CBA mouse islet grafts harvested from BALB/c recipients.**

A) H+E stained section of the renal sub-capsular space which received pPSC1 (vector alone) transfected islets. The graft rejected at 10 d and the kidney was harvested at 12 d.

B) H+E stained section of a CTLA4-Ig transfected islet allograft harvested from a normoglycemic mouse at 130 d post-transplantation. Mononuclear cells are present in dense aggregates adjacent to the islets, but are clearly not infiltrating the allograft.

C&D) Immunohistochemical staining for insulin (brown) in CTLA4-Ig transfected allografts harvested from normoglycemic mice at 50 d and 130 d respectively. Note that different sections of the same graft are shown in B and D.

E&F) Sections from a CTLA4-Ig transfected allograft harvested from a normoglycemic mouse at 164 d, cut at two different tissue levels and stained for insulin. Variable numbers of mononuclear cell aggregates are shown in E and F, within different areas of the allograft. The graft is from an animal which did not become hyperglycemic post-nephrectomy -- note that there is still a significant number of β-cells present.

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CHAPTER IV

OPTIMIZATION OF THE BIOLISTIC TRANFECTION PROCESS IN MOUSE ISLETS.

INTRODUCTION

The term biolistics (derived from the words biological and ballistics) was coined by the inventors of the process, E D Wolf, N K Allen and J C Sanford, in 1984 (*1*). The process has also been referred to as the particle gun method, the microprojectile method, the gene gun method, the particle acceleration method and the bio-blaster method. In all cases, it is used to describe a process whereby microparticles travelling at high velocity are used to deliver substances into cells.

With the evolution of molecular techniques which allowed gene cloning, DNA sequencing and synthesis, the ability to deliver genes into cells has also become of utmost interest. As previously discussed, many processes have become available, each with their inherent advantages and disadvantages. An ideal gene delivery system would not only allow simple, rapid transformation of easily transformable cells, but also allow transformation of cells which have not been able to be transformed by other currently available techniques.

As a plant geneticist, Sanford was initially looking for a method to transform plant pollens (*2*). The biolistic methodology has now evolved to also allow transformation of prokaryotic bacteria (*3, 4*) and microbial eukaryotes such as yeast and fungi (*5*) and algae (*1*). Genomes of subcellular organelles such as chloroplasts (*6, 7*) and mitochondria (*8, 9*) have also been biolistically transformed. The process has more recently been extended to include animal cell lines (*10*), primary animal cells (*11*), and even intact animals(*12*) (*13*).

The advantages of biolistic transfection of pancreatic islets are several. First, islets have proven to be difficult to transfect using classical chemical methods. An islet is a unique structure, composed of a dense cluster of disparate endocrine cell types surrounded by a capsule of connective tissue. Therefore, with the exception of viruses, it is difficult for the DNA to gain access to the cellular membranes of the individual cells. Second, biolistics is a non-viral transfection method. In as much as our ultimate goal is to use transfected islets in a transplant model to abrogate graft rejection, we do not want to introduce any kind of additional immunological stimulus into our system. Third, plasmid DNA, either singly or in combination, can be used directly for transfection. That is, there is no additional, lengthy preparation time after cloning, such as in recombinant adenovirus preparation.

1. Principle.

The biolistic process revolves around acceleration of DNA-coated particles to high velocity. Several methods of particle acceleration were initially assessed by Sanford's group (*14*). These included a simple air blast, use of centripetal acceleration, electrostatic acceleration, and the use of a 'macroprojectile' which would accelerate microparticles on its surface. The latter proved to be the most successful and underwent further development. The macroprojectile is driven to supersonic speed by means of a gas shock wave. This gas shock can be derived from the explosion of gunpowder (*15*), an electric explosion of a water droplet (*16*), a blast of compressed air (*17*) or the release of a helium shock wave following rupture of a membrane (*14*). There are several advantages of helium driven units over the other models. Helium is a much cleaner power source than gunpowder, which generates dirty debris within the apparatus. In addition, it is safer, and can be regulated at the source. Being a light gas, it expands quickly which means that it can accelerate particles to a higher velocity than can compressed air or nitrogen.

The macrocarrier can be composed of a high density polyethylene cylinder (DuPont gunpowder models), hollow polyethylene tubing (Agracetus model) or a circular Kapton membrane (BioRad model). In essence, the macrocarrier simply has to provide a surface to carry the microcarrier particles on, and to be able to withstand the gas shock, sudden acceleration and abrupt deceleration (in the case of DuPont and BioRad models) which is inherent to the process.

Regardless of the apparatus used, the biolistic blast is generally released under vacuum since the microprojectiles are rapidly decelerated as they pass through a gas. This is increasingly the case as the particles become smaller. Also, by replacing the denser air by a low molecular gas such as helium, the shock wave produced may be potentially less damaging to the exposed tissue. In most units, therefore, the entire apparatus, including the tissue to be exposed, is contained within a vacuum chamber.

There are essentially two types of microparticles which are used for biolistics. Tungsten was the substance initially used, but the particles are extremely irregular in shape and heterogeneous in size, which prevents optimization of particle size for different cell types. Other disadvantages are the fact that it is potentially toxic to some types of cells, it is subject to surface oxidation which can affect DNA binding, and it catalytically degrades DNA which is bound to it over time (*14*). The advantages of tungsten are that it is inexpensive and easy to coat with DNA. Gold particles are currently the microprojectiles of choice, their major disadvantage being the expense. They are also not stable in aqueous suspensions, and agglomerate irreversibly over time. The DNA coating process seems to be more variable, and is affected by atmospheric humidity. The advantages of using gold are that the particles are round, relatively uniform in size, and are available in a range of diameters. This allows optimization of particle size for specific cell types. The most important advantage of gold, however, is the fact that it is a biologically inert substance. Gold has not been found to be toxic to any type of cell tested (*14*), and is approved by the appropriate regulatory agencies as a therapeutic agent in humans.

In our studies we have used a helium-driven macrocarrier disc system, and gold microparticles. Our unit is a hand-held prototype manufactured by BioRad, to which we have made a number of modifications. A schematic of the apparatus appears in Figure 4-1.

Basically, high pressure helium is restrained within a small cylinder by a membrane. The gold microprojectiles are coated with the DNA of interest, and then applied to the undersurface of a macrocarrier disc. The islet tissue is placed on the upper surface of an inverted cell strainer, at some distance directly below the macrocarrier disc. The entire system is encased within a mini vacuum chamber. Upon firing, the restraining membrane is either spontaneously ruptured by the increasing helium pressure, or by a manually-activated lance. This results in release of a high pressure helium shock wave which drives the macrocarrier disc at high velocity down onto a stopping screen (just above the islets) where it is arrested. The microprojectiles, however, continue at high velocity through the gaps in the stopping screen and bombard the tissue below. Following tearing through the cellular membrane, some particles come to rest within the cytoplasm of the cell. It is possible that others may pass right through a cell, although the density of the cytosol should decelerate the particles significantly. Other particles may not hit any islet tissue at all, and simply come to rest in the liquid medium below. The latter event is unique to our system since the islets are discrete, floating entities. In the case of a continuous monolayer of cells, all particles would bombard a cell (see Discussion).

2. Optimization.

Having shown that biolistic transfection of intact islets was possible (Chapter II), we decided to investigate the procedure more closely, in order to optimize the conditions and therefore achieve as efficient islet transfection rates as possible. There are a number of interactive parameters which affect the velocity of the macrocarrier disc and ultimately, the velocity of the microprojectiles. These include the pressure of the gas source (helium), the gap distance (distance from the rupture disc to macrocarrier), the macrocarrier flight distance, and the target distance (distance from the stopping screen [i.e. microprojectile launch site] to the target tissue). Obviously, higher pressures, shorter gap distances and longer macroprojectile flight distances all result in higher launch velocities. A longer microprojectile flight distance would result in lower impact velocity, but results in better particle dispersion and less cellular damage due to the shock wave. Additionally, smaller microprojectiles or decreased vacuum pressures would result in lower impact velocities.

In most chamber models, many of these parameters can be altered. However, in our model all of the distances between the elements are fixed. We therefore chose to consider the following three variables: the number of islets exposed per biolistic blast, the power source helium pressure, and the size of the gold particles used. In addition, we assessed the transfection efficiency using two different sample loading techniques. In all cases, firefly luciferase driven by the human Immediate Early CMV promoter was used as a reporter gene. Once the optimal conditions had been determined, a series of blasts were performed to determine the reproducibility of the procedure.

MATERIALS AND METHODS

1. Islet isolation.

BALB/c mice were anesthetized using tribromoethanol (Avertin). Pancreatic islets were isolated using collagenase digestion (*18*), and purified on a dextran gradient followed by hand-picking. The islets were cultured overnight in 95% air/5% CO_2 at 37°C in Ham's F10 media supplemented with 14.3 mM $NaHCO_3$, 10 mM glucose, 2 mM L-glutamine, 10 mM nicotinamide, 0.5% bovine serum albumin, 50 mM IBMX (isobutyl-methyl-xanthine), 100 U/mL penicillin and 100 μg/mL streptomycin.

2. Molecular constructs.

The firefly luciferase gene driven by the human Immediate Early CMV promoter/enhancer element in a pBR322-derived plasmid (kindly supplied by Dr. D. Drucker, University of Toronto) was used as a reporter construct in all cases.

3. Biolistic transfection.

Gold particles (BioRad) were used as microprojectiles and were found to have good uniformity by scanning electron microscopy (Hitachi S-2500, see Chapter II). Particles (30 mg) were washed initially with 95% ethanol, twice with sterile water, then pelleted and resuspended in 500 µL of sterile water (final concentration of 60 mg/mL). Each disc was prepared using a separate DNA precipitation reaction, and the entire reaction mixture was loaded onto the macrocarrier disc. The DNA precipitation onto the gold particles was achieved by adding 3.3 µg (2.4 µL) of DNA (1.4 µg/µL) to 8.3 µL of Au particles (60 mg/mL), followed by 10.7 µL of 2.5 M $CaCl_2$ (i.e. a volume equal to the volume of DNA and Au particles) and 2.1 µL of 1 M spermidine (1/5 of the volume of $CaCl_2$ used). The mixture was vortexed, left to settle at room temperature for a minimum of 10 min, and spun in a microcentrifuge briefly. The pellet was washed with 70% ethanol, re-pelleted, washed with 95% ethanol, re-pelleted and resuspended in 10-15 µL of 95% ethanol. The central area (~7.5 mm in diameter) of a 2.54 cm in diameter, 0.06 mm in thickness Kapton macrocarrier disc (BioRad) was then covered with the particle slurry (~3 µg DNA) and allowed to dry in a desiccator. For exposure to the biolistic blast in the islet number, helium pressure and particle size studies, the islets were suspended in 0.10 mL of media on the top of an inverted cell strainer with a mesh size of 40 µm (Falcon). The strainer and blasting apparatus was encased in a small vacuum chamber, (a modification to a hand-held version of the BioRad biolistic apparatus) of 15" Hg. Using this system, the target distance between the stopping screen and the suspended cells was 25 mm. Following the blast, the islets were resuspended in Ham's F10 media supplemented with 14.3 mM

$NaHCO_3$, 10 mM glucose, 2 mM L-glutamine, 10 mM nicotinamide, 0.5 % BSA, 50 mM IBMX (isobutyl-methyl-xanthine), 100 U/mL penicillin and 100 µg/mL streptomycin, and incubated at 37°C in 95% air/5% CO_2. Control groups were put directly into culture.

In Study 1, an examination of the effect of different numbers of islets being exposed to each blast, groups of 250, 500 or approximately 1000 islets were used per blast, along with 1.6 µm particles and spontaneous disc rupture at a pressure rating of 400 psi of helium. On the basis of the results obtained, all further studies were carried out using 250 islets per blast. In Study 2, a study of the effects of helium source pressure, 1.6 µm particles were used. As the rupture discs obtained from BioRad were rated to spontaneously rupture at a pressure of 400 psi, a source helium pressure of 400, 800 or 1200 psi of helium was obtained simply by using either one, two or three rupture discs respectively. In all cases, the helium pressure at the tank was set to 200 psi greater than the pressure required for disc rupture. Study 3, the effect of gold particle size, used spontaneous disc rupture at a pressure rating of 400 psi of helium and gold particles of 1.6, 2.2, 3.1 or 4.0 µm in diameter. The same weight of gold particles (0.5 mg) and amount of DNA (3.3 µg) was used for DNA precipitation in all cases. Study 4 compared the effect of loading the islet sample in two different ways. In both cases, the islets were initially resuspended in 0.1 mL of media. In the first case, the islets were placed on a dry cell strainer, where they remained within the liquid drop (sitting drop). In the second case, the cell strainer was first loaded with 0.1 mL of media, which was then removed. When the islets were then loaded onto the pre-wet cell strainer, the liquid drop flowed through the mesh and became suspended on its bottom surface, while the islets remained on the upper surface of the mesh (hanging drop).

4. Reproducibility of blasting procedure.

In order to check the reproducibility of the blasting procedure itself, two series of 4 blasts each was done using the optimal conditions ascertained by the above studies.

Uncoated 1.6 µm gold particles were loaded onto macrocarrier discs in the regular fashion. The gold particles were blasted either on to cell strainers or Petri dishes which had black electrical tape attached to them, sticky side up. Photographs were taken to document the appearance of the rupture and macrocarrier discs before and after the blast, as well as the distribution of the particles on the tissue-supporting surface.

5. Viability staining.

In the studies of helium pressures and particle sizes, a small representative sample of islets was removed just before the islets were harvested for the luciferase assays. A stock solution of fluorescein diacetate (Sigma, 0.1 mM in acetone) was diluted 1:100 with Hank's buffered salt solution. A further 1:10 dilution was made through addition of the cells in media, resulting in a final fluorescein diacetate concentration of 10 µM. Since fluorescein diacetate is a nonpolar compound, it readily transverses the bipoloar membrane. Once inside a viable cell, it is hydrolyzed by esterases to form a polar green fluorescein product, which accumulates within the cell. Therefore, the degree of green fluorescence given off by the stained islets is a qualitative indication of the viability of the cells.

6. Luciferase assays.

To determine transfection efficiency, islets were harvested after 48 h of culture, counted and washed 3 times with fresh media. The resulting pellet was mixed with 50 µL of cell lysis buffer consisting of 0.1 M Tris pH 7.8, 0.5% NP 40, and 1.0 mM dithiothreitol (DTT). The lysate was spun in a microcentrifuge x 16,000 g for 15 min at 4°C. Luciferase enzyme activity was measured immediately on a Lumat LB 9501 (Berthold) using 20 µL of cellular lysate in 100 µL of assay buffer consisting of 0.5 mM luciferin in 20 mM Tricine, 1 mM $MgCO_3$, 2.7 mM $MgSO_4$, 0.1 mM EDTA, 0.03 M DTT, 0.5 mM ATP, 0.3 mM Coenzyme A, and 1 ng/mL bovine serum albumin (Sigma Fraction V), final pH 7.8. Results are expressed as relative light units (RLU)/islet.

RESULTS

1. Effect of the number of islets exposed to the biolistic blast.

Since the islets were suspended in a droplet rather than arranged in a monolayer, we were concerned that the transfection efficiency may be affected by the density of the islets within the droplet. To test this, groups of either 250, 500 or approximately 1000 islets were used in each separate blast. The results obtained are shown in Table 4-1. The mean % recovery of islets when either 250, 500 or approximately 1,000 islets were exposed per blast is identical. Although mean values of RLU/islet appear to be quite different, there is no statistically significant difference between them, due to the large variation in values within each group. On the basis of these findings, it was decided that all further investigations could be carried out using 250 islets per blast.

2. Effect of different helium pressures per blast.

One of the few variables which can be changed using our system, is the pressure of the helium used to accelerate the gold particles on the macrocarrier disc. The results obtained using a helium source pressure of 400, 800 and 1200 psi are shown in Table 4-2. Although it appears that the % recovery of intact islets is less when a helium pressure of 1200 psi was used (33% compared to 55% and 51%), there is no statistically significant difference between the groups. In contrast, the amount of enzyme activity produced from the transfected gene (RLUs/islet) is significantly higher at the lowest helium pressure of 400 psi (1220 RLU/islet versus 55 RLU/islet and 114 RLU/islet at 800 and 1200 psi respectively).

The fact that higher helium pressures are more damaging to the islets is demonstrated in Figure 4-2. Islets blasted with a helium pressure of 400 psi appear intact, with a uniform pattern of intense fluorescence, indicating the presence of viable cells (Fig 4-2A). With an increase of helium pressure to 800 psi, the islets begin to have a somewhat moth-eaten appearance due to patches of non-viable cells (Fig 4-2B), and with 1200 psi there has been extensive damage to some islets, with very little viable tissue remaining (Fig 4-2C).

3. Effect of gold particle size.

Another parameter that can be varied in the biolistic procedure is the size of gold particle used as the microcarrier. BioRad provided us with uniformly-sized particles. In our studies we evaluated the effect of particles measuring 1.6, 2.2, 3.1 and 4.0 μm in diameter on both gene expression and cell viability. The results appear in Table 4-3. There is no significant difference in the number of islets recovered when different particles sizes were used. Although the results are not statistically different due to the large range of values obtained, in most cases there appears to be substantially more luciferase expression with the 1.6 μm particles. Similarly, the fluorescence staining for viability results show a progressive increase in cell damage with an increase in particle diameter, as shown in Figure 4-3. When 4.0 μm particles are used, for example, some islets show only a small rim of remaining viable tissue on one side, or a few small islands of viable tissue (Fig 4-3D). On the basis of these results, we elected to use 1.6 μm particles in all further studies since this meant we would be using a greater number of particles per blast, and thus targeting more islet cells. In addition, the 1.6 μm particles are now commercially available which influenced our choice for purely practical reasons.

4. Effect of sample loading procedure.

During our studies, we noted that there appeared to be a difference in gene expression depending on whether the islets remained within a drop of media sitting on top of the cell strainer, or whether the media flowed through and hung below the strainer leaving the islets relatively dry on the top of the mesh. All of the above studies were performed with the islets suspended within the media drop sitting on top of the strainer.

The results shown in Table 4-4 were obtained when a comparison was made of the two sample loading procedures, using 1.6 µm particles and a helium pressure of 400 psi. There is no significant effect of the different loading procedures on islet recovery. There is, however, significantly more gene expression when the islets were placed on a pre-wet strainer, meaning that the media was hanging in a drop beneath the strainer. In this case, the mean RLUs/islet obtained is 18 times greater than that obtained when the islets were blasted within a drop of media sitting on top of the cell strainer.

An important point to note is that there is always a large variation in the values obtained within each group of study parameters, regardless of the factor being examined. Interestingly, there seems to be a fairly consistent difference of a factor of approximately 25 between the highest and lowest value of each of the optimal factors groups (250 islets/blast, 400 psi of helium, 1.6 µm gold particle, hanging drop of media). To superficially compare the blast-to-blast reproducibility of the blasting procedure itself, a series of blasts was performed using uncoated gold particles onto black tape attached to either cell strainer or Petri dish targets. The results are shown in Figures 4-4 and 4-5 respectively. In both cases, variability is evident in both the apparent amount and the distribution of gold particles on the macrocarrier disc prior to firing. Further, a random, often eccentric pattern of gold particles is obtained on both types of target following the blast. In some cases the eccentricity seems to result from the rupture disc initially bursting on one side rather than centrally (e.g. Figure 4-4, Sample 3). In some cases the macrocarrier disc is seen to have hit the stopping screen eccentrically (e.g. Figure 4-5, Sample 3). In one case, the disc appears to have hit the stopping screen in a folded manner (Figure 4-5, Sample 4).

DISCUSSION

Our first study's purpose was to determine whether there was any difference in gene expression when different numbers of islets were exposed per blast. Happily, there was no significant difference in either islet recovery or gene expression with the different numbers of islets used. Therefore, we elected to use 250 islets per biolistic exposure for the remainder of the studies. The result was that we could get much more information per islet isolation and could cut down considerably on isolation expenses and the number of animals used for the optimization studies. However, with fewer islets, many more particles don't hit an islet at all during the bombardment process and are therefore simply wasted.

The rupture discs that we obtained from BioRad were manufactured so that each disc would rupture when a gas pressure of 400 psi was reached. Discs of differing rupture pressures were made by binding multiples of the discs together and were therefore available at rupture pressures which were multiples of 400. In our original islet studies, the helium tank pressure was set to 1200 psi, and a disc with a rating of 1600 psi was ruptured with a manually-operated lance 2-3 seconds after the helium had been turned on (see Chapter II). Due to the design of our machine, the pressure valves, the length of the helium lines and the way they entered into the apparatus, we later realized that 2-3 seconds was not long enough for the pressure to have reached maximum, and that the helium pressure at the rupture disc would have been considerably less than 1200 psi. For this reason, in our optimization studies we decided to use spontaneous rupture of the discs, which would ensure the pressure of the released shock wave. Rupture pressures of 400, 800 and 1200 psi were obtained by using either 1, 2 or 3 discs (400 psi each) respectively. Our data clearly show that the lowest helium pressure of 400 psi results in the best gene expression. Unfortunately, due to the nature of the discs, we are not able to go any lower than 400 psi using this spontaneous rupture procedure. It may well be that an even lower pressure is optimal. Our results show that the number of recovered islets is less at higher helium pressures, and the fluorescent stains also show a progressive loss of viability with an increase in helium pressure. This is most likely due to the damaging effects of the shock wave itself to all of the exposed islets, rather than due to the velocity at which the particles bombard a small proportion of cells. In fact, it has been our experience that if islets are exposed to the shock wave twice, even without particles, there is extensive damage to the islets.

The optimal size of the gold particle to be used should be ascertained for each tissue system. In our islet studies, there was no significant difference between the different particle sizes, although there was a trend for smaller particles to be better. This was also reflected in the fluorescent staining, where there appeared to be more tissue damage with the larger particles. A smaller particle would have less impact velocity and one would intuitively expect that there would be less overall damage since the holes in the target membrane would be smaller. Note that the weight of gold used for particle preparation is the same (30 mg), regardless of the size used. This means that fewer particles are present with the larger diameters. An increased surface area may have compensated for this in terms of the amount of total DNA precipitation, resulting in similar amounts of foreign protein being produced in each group. However, the fact remains that fewer cells are targeted when larger particles are used. For the above reasons and the availability considerations, we elected to use 1.6 μm gold particles in all further studies.

Most of the previous experience using biolistic transfection has been with monolayers of cells comprising the target tissue. Our system is unique in that our tissue targets are large, discrete clusters of cells, some of which may even be overlapping during the bombardment procedure. In general, if the islets are suspended within a droplet, it has been our experience that they tend to cluster towards the centre of the drop. On the other hand, if they are well mixed before loading onto a pre-wet cell strainer, the media quickly flows through the mesh and the islets appear to be well distributed on the top of the membrane. Figure 4-6 illustrates the various possibilities when different tissue distributions are bombarded by the spray of DNA-coated gold particles. In fact, during the course of our studies, we had observed that on the occasions when the media flowed through the mesh and hung on the bottom, (and the data was therefore excluded from the study), there was a trend of higher gene expression. When the two sample loading procedures were formally compared, there was significantly more gene expression using the pre-wet strainer and hanging drop of media, with no adverse effect on islet recovery.

On the basis of all of the aforementioned studies, and given the limitations of our apparatus, the most optimal conditions for biolistic transfection of intact, isolated murine islets were determined to be:

1. Spontaneous disc rupture at 400 psi of helium.
2. Gold particles of 1.6 μm diameter.
3. Sample loading onto a pre-wet strainer with flow-through of the media.

These conditions will therefore be used in future studies of biolistic islet transfection with immunomodulatory molecules.

Unfortunately, despite careful attention to performing the entire transfection procedure as carefully and consistently as possible, there is still a large variability in transfection rates obtained. This is mostly due to the lack of reproducibility inherent in the machinery itself, but is also a result of the nature and architecture of the target tissue we are using, namely intact islets. Despite this drawback, we feel that biolistics offers an easy, safe and relatively efficient way to transfect this primary tissue which is resistant to most other transfection methodologies.

Table 4-1. The effect of the number of islets exposed per biolistic blast.

# per Blast	# Recovered	% Recovery	Total RLUs	RLUs/islet
1000	850	85	111193	131
800	520	65	13048	25
1075	650	60	74962	115
1000	650	65	238655	367
1000	570	57	4796973	8416
Mean (n=5)		66		1811
500	230	46	37611	164
500	360	72	49273	137
500	350	70	7885	23
500	280	56	164335	632
500	320	64	71724	224
500	425	85	11867	29
Mean (n=6)		66		202
250	180	72	50085	278
250	135	54	37942	281
250	200	80	29752	149
250	200	80	19444	97
250	210	84	29671	141
250	61	24	64456	1057
250	140	56	170572	1218
250	185	74	20773	112
250	165	66	411283	2493
Mean (n=9)		66		647

$p > 0.05$ between all groups, single factor ANOVA

Table 4-2. The effect of different helium pressures per blast.

Helium (psi)	# Recovered	% Recovery	Total RLUs	RLUs/islet
400 psi	61	24	64456	1057
400 psi	140	56	170572	1218
400 psi	185	74	20773	112
400 psi	165	66	411283	2493
Mean (n=4)		55		1220 *
800 psi	76	30	6027	79
800 psi	135	54	0	0
800 psi	155	62	20158	139
800 psi	140	56	0	0
Mean (n=4)		51		55
1200 psi	63	25	16389	263
1200 psi	90	36	847	9
1200 psi	105	42	0	0
1200 psi	90	36	1815	23
1200 psi	60	24	13722	274
Mean (n=5)		33		114

* $p < 0.05$, single factor ANOVA followed by Tukey's post hoc comparison

Table 4-3. The effect of gold particle size.

Diameter (μm)	# Recovered	% Recovery	Total RLUs	RLUs/islet
1.6	180	72	50085	278
1.6	135	54	37942	281
1.6	200	80	29752	149
1.6	200	80	19444	97
1.6	210	84	29671	141
1.6	61	24	64456	1057
1.6	140	56	170572	1218
1.6	185	74	20773	112
1.6	165	66	411283	2493
Mean (n=9)		66		647
2.2	180	72	3333	19
2.2	235	94	83137	387
2.2	103	41	3787	37
2.2	230	92	42	0
Mean (n=4)		75		111
3.1	105	42	0	0
3.1	155	62	0	0
3.1	185	74	0	0
3.1	160	64	20076	134
3.1	215	86	9	0
Mean (n=5)		66		27
4.0	60	24	6578	110
4.0	195	78	3216	17
4.0	140	56	319	2
4.0	140	56	10232	73
Mean (n=4)		54		50

$p > 0.05$ between all groups, single factor ANOVA

Table 4-4. Comparison of sample in hanging drop vs. sitting drop.

Sample Drop	# Recovered	% Recovery	Total RLUs	RLU/isl
Sitting	180	72	50085	278
Sitting	135	54	37942	281
Sitting	200	80	29752	149
Sitting	200	80	19444	97
Sitting	210	84	29671	141
Sitting	61	24	64456	1057
Sitting	140	56	170572	1218
Sitting	185	74	20773	112
Sitting	165	66	411283	2493
Mean (n=9)		66		647
Hanging	165	66	3535171	21425
Hanging	187	75	2477635	13249
Hanging	110	44	522674	4752
Hanging	175	70	581743	3324
Hanging	110	44	127553	1160
Hanging	160	64	5055830	31599
Hanging	125	50	811010	6488
Mean (n=7)		59		11714 *

* $p < 0.05$, Student's t-test

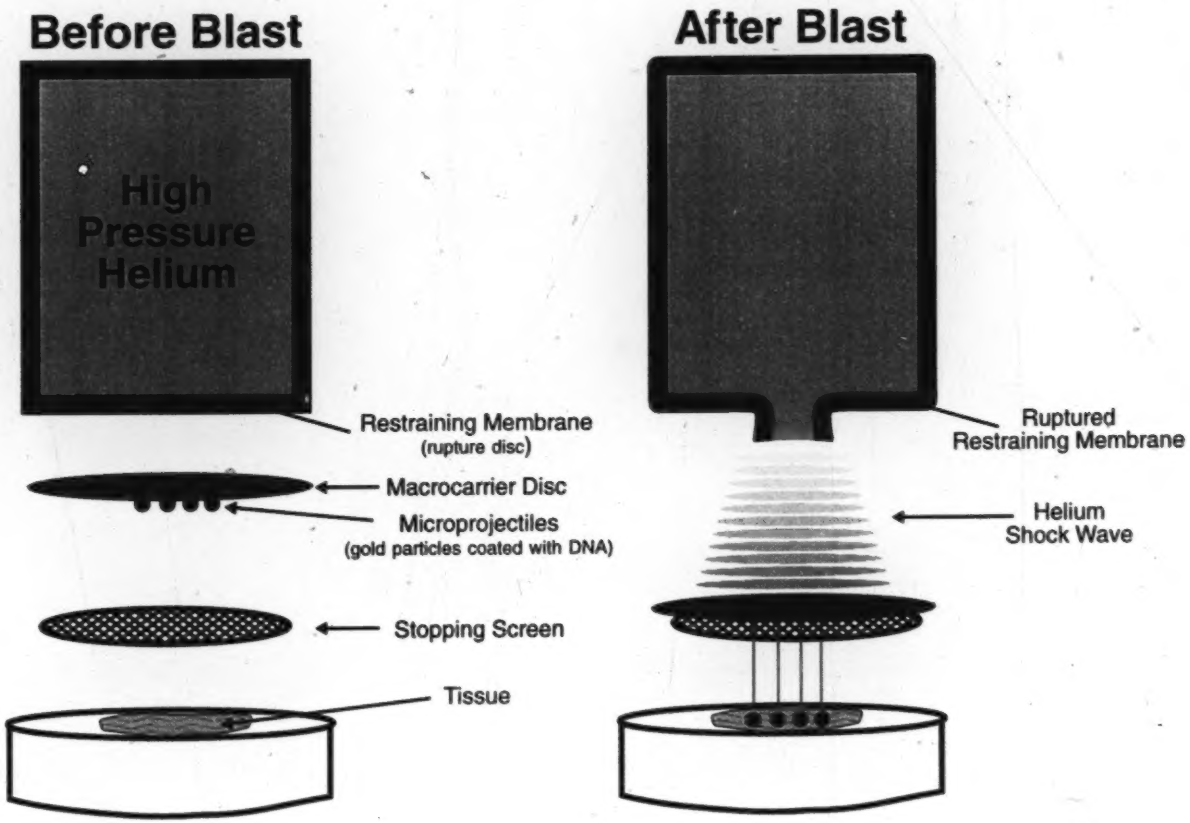
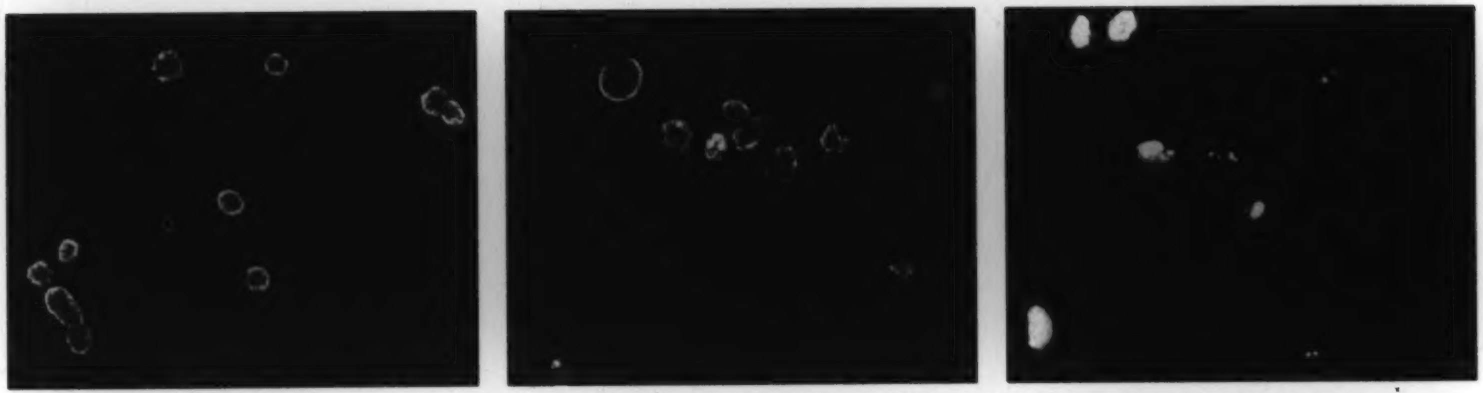
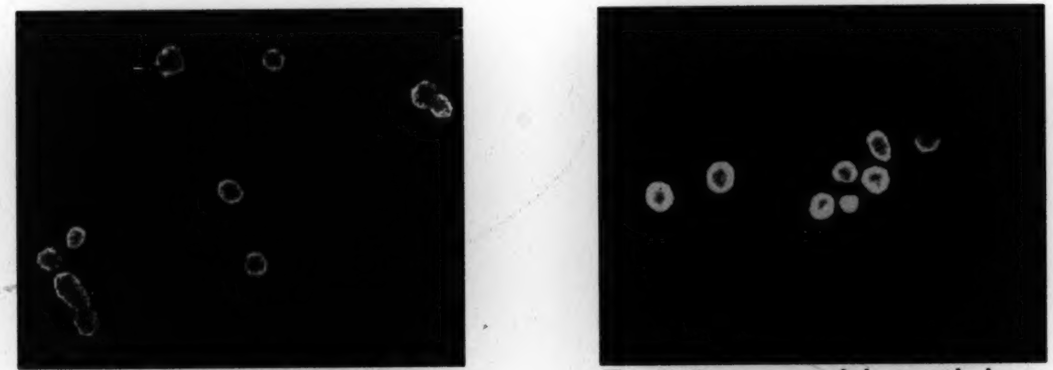

Before Blast
High Pressure Helium
Restraining Membrane
(rupture disc)
Macrocarrier Disc
Microprojectiles
(gold particles coated with DNA)
Stopping Screen
Tissue
After Blast
Ruptured Restraining Membrane
Helium Shock Wave

Figure 4-1. Principle of biolistic transfection procedure.

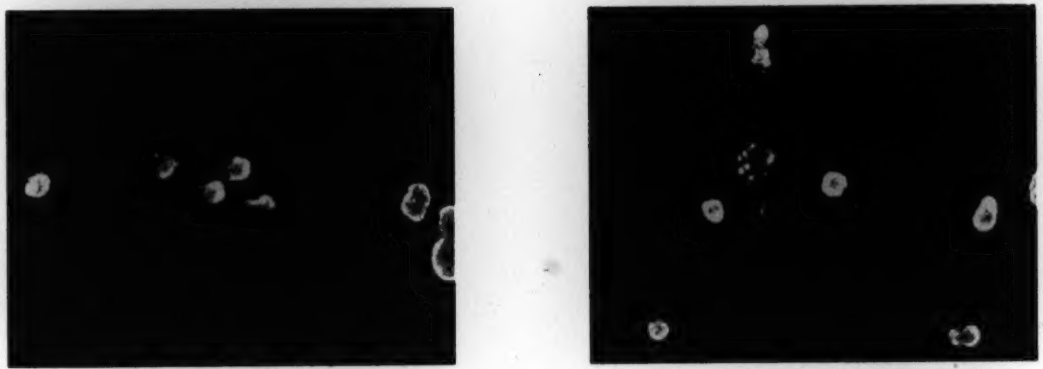


A. 400 psi helium **B.** 800 psi helium **C.** 1200 psi helium

Figure 4-2. **The effect of helium pressure on viability of blasted islets.**
Islets have been stained with fluorescein diacetate and examined under ultraviolet light following blasting using different helium source pressures. Viable cells are highly immunofluorescent.



A. 1.6 µm gold particles B. 2.2 µm gold particles



C. 3.1 µm gold particles D. 4.0 µm gold particles

Figure 4-3. **The effect of gold particle size on viability of blasted islets.**
Islets have been stained with fluorescein diacetate and examined under ultraviolet light following blasting with gold particles of different diameters. Viable cells are highly immunofluorescent.

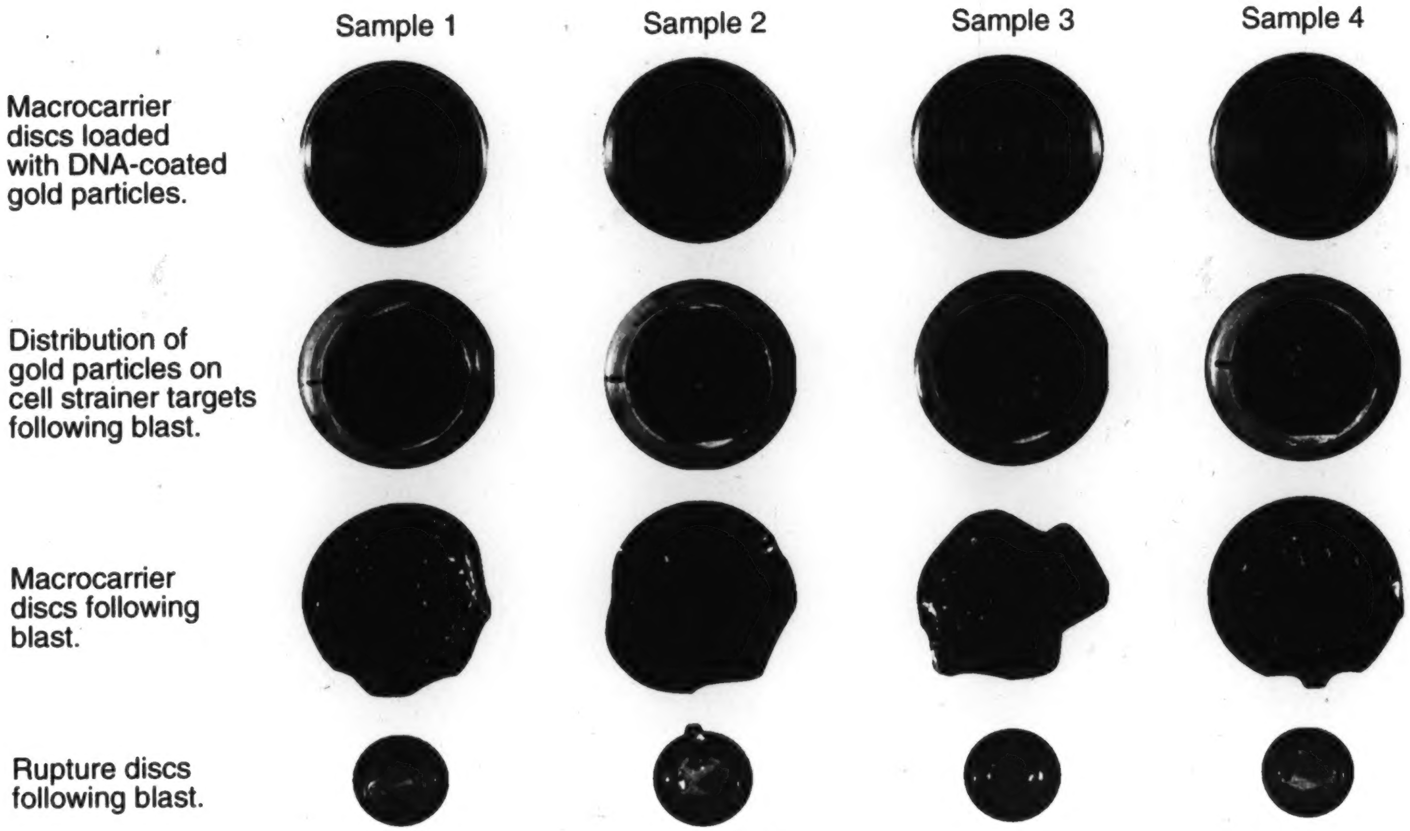

Sample 1
Sample 2
Sample 3
Sample 4
Macrocarrier discs loaded with DNA-coated gold particles.
Distribution of gold particles on cell strainer targets following blast.
Macrocarrier discs following blast.
Rupture discs following blast.

Figure 4-4. **Reproducibility of biolistic particle acceleration onto cell strainer targets.**

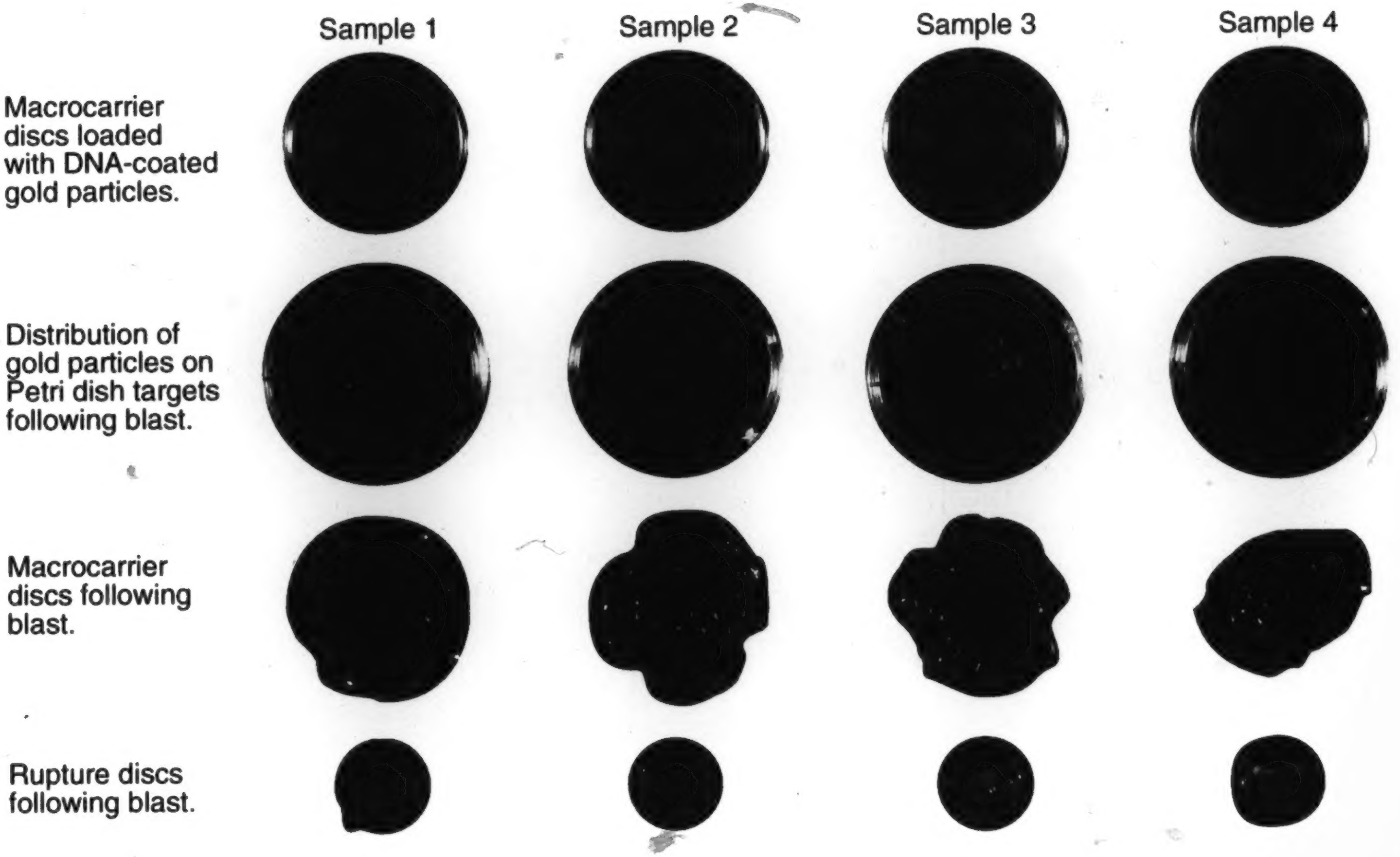
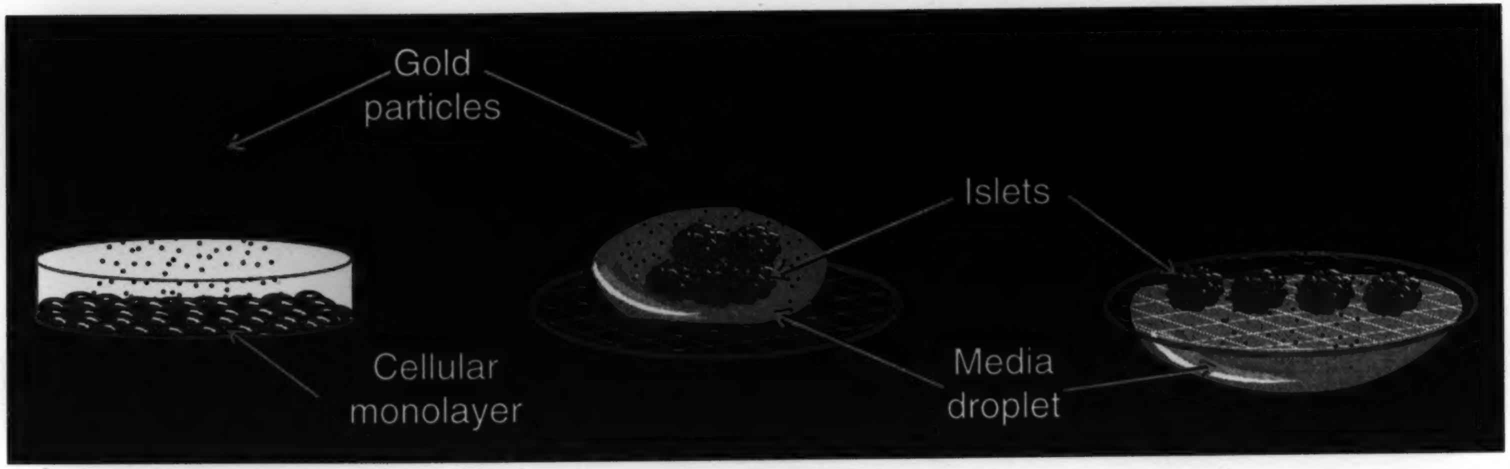

Sample 1
Sample 2
Sample 3
Sample 4
Macrocarrier discs loaded with DNA-coated gold particles.
Distribution of gold particles on Petri dish targets following blast.
Macrocarrier discs following blast.
Rupture discs following blast.

Figure 4-5. **Reproducibility of biolistic particle acceleration onto Petri dish targets.**


Gold particles
Islets
Cellular monolayer
Media droplet

Confluent Cellular Monolayer	Centrally Clumped Islets	Separated Islets
● All particles will bombard a cell ● Some cells may contain >1 particle	● Many particles will not bombard an islet ● Several islets may not be bombarded	● Many particles will not bombard an islet ● Most islets will be bombarded

Figure 4-6. **Patterns of gold particle bombardment with different tissue loading procedures.**

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CHAPTER V

PROLONGATION OF THE SURVIVAL OF BIOLISTICALLY TRANSFECTED MOUSE ISLET ALLOGRAFTS EXPRESSING HUMAN CTLA4-Ig, HUMAN SOLUBLE FAS LIGAND OR A COMBINATION OF HUMAN CTLA4-Ig AND HUMAN SOLUBLE FAS LIGAND.

INTRODUCTION

The aim of this study was to attain immunosuppression localized to a limited area around engrafted allogeneic islet tissue. We hoped to achieve this by transfecting the transplanted islets with genes for immunomodulatory molecules. CTLA4-Ig is a good candidate molecule for this therapy in allograft transplantation since it should result in blockade of the co-stimulatory signal which is generally required for T cell activation (see Chapters I and III). Indeed, we have previously shown that when mouse islets were biolistically transfected with human CTLA4-Ig, long-term allograft survival was the result in 50% of cases (*1*). We attributed the lack of uniform long-term survival mainly to the failure of the biolistic transfection process to consistently achieve adequate levels of protein expression. With subsequent technical modifications, particularly to the sample loading procedure, we were able to improve our protein expression levels approximately 20 fold. We therefore decided to investigate whether we could improve our rate of success of islet allograft survival with CTLA4-Ig under these new conditions.

We also elected to use the biolistic methodology to introduce a second promising immunoregulatory molecule, Fas ligand. The rationale for its use was discussed previously (Chapter I). Briefly, interaction of Fas ligand with a Fas receptor-bearing target cell results in apoptosis of the target (*2*). Fas receptor/Fas ligand interactions are thought to play an important role in the regulation of clonal expansion and subsequent limitation of T cell responses (*3, 4*). Activation of T cells in vitro initially results in upregulation of their Fas receptor expression (*5*). Lymphocyte Fas ligand expression is also upregulated (*6-8*). Interaction of the Fas receptor with Fas ligand then triggers the Fas receptor-bearing T cell to undergo apoptosis. It is generally believed that this process curtails immune responses and limits the size of the remaining antigen-specific populations (*4*).

In the transplantation setting, the rationale for use of Fas receptor/Fas ligand interactions to disrupt the allograft rejection process is as follows. T cells infiltrating the graft should have high levels of Fas receptor expression due to allo-activation (*9*). Soluble Fas ligand, produced by the engrafted tissue, should bind to the Fas receptors on the activated infiltrating lymphocytes and trigger apoptosis. The consequence would be survival of the allograft.

In the following study we have used biolistically-transfected mouse islets to examine the effect of locally produced human CTLA4-Ig alone, human soluble Fas ligand alone and the combination of the two molecules on mouse islet allograft survival.

MATERIALS AND METHODS

1. Islet isolation.

CBA mice (Jackson Laboratories, Bar Harbor, Maine) were anesthetized using tribromoethanol (Avertin). Pancreatic islets were isolated by shaking collagenase digestion and purified on a Ficoll density gradient followed by hand-picking. The islets were cultured for 24 h in 95% air/5% CO_2 at 37°C in RPMI 1640 media supplemented with 12 mM HEPES, 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 20 µg/mL amphotericin B. Islets from each isolation were hand-picked into aliquots of 500 and cultured under the same conditions for a further 24 h. Similar methods were used in the case of the syngeneic controls, except that the islet donors were BALB/c mice.

2. Molecular constructs.

The cDNA encoding human CTLA4-Ig was modeled essentially on the construct of Linsley et al. (*10*). Our construct contained three DNA segments encoding the following elements: 1) the oncostatin M signal sequence (amino acids 1 to 25), 2) the extracellular domain of human CTLA4 (amino acids 26 to 150), and 3) the human Fcγl region, with cys to ser substitutions for all cys residues in the hinge region (amino acids 151 to 383). These substitutions result in disruption of the disulfide bridges between the two strands of the Ig tail and are thought to also destabilize the complement binding sites of the Fc region. Specific restriction sites which did not affect the encoded amino acid sequence were incorporated between the three elements to allow substitutions in the future if desired (see Appendix for cloning details). The complete cDNA construct was sequenced and was verified as correct with the introduced restriction sites with one exception. A non-coding point mutation of thymidine for guanosine occurred at nucleotide position 726, within the region encoding for the immunoglobulin tail. The complete gene sequence appears in the Appendix. The cDNA encoding the fusion protein was excised with Xba I and Not I and inserted into a pUC-based eukaryotic expression vector (pPSC1) which contained the human CMV (Towne strain) Immediate Early 1 promoter/enhancer + Intron A as well as a downstream polyadenylation signal from SV40 virus (J.F.E., unpublished). The CTLA4-Ig protein produced by this construct bound to a cell line transformed with B7, as determined by flow cytometry. Since our construct was virtually identical to that produced by Peter Linsley (who has extensively characterized the molecule), no further in vitro functional tests of the molecule were done.

The cDNA encoding human soluble Fas ligand had been previously cloned in the laboratory of Dr. J.F. Elliott by Dr. Camille Hancock-Friesen (*11*). It was modeled on the construct of Nagata's group (*12*), and consisted of a truncated form of the wild-type molecule which only encoded the extracellular portion of the Fas ligand molecule, preceded by the signal sequence for murine granulocyte colony stimulating factor (G-CSF). The complete gene sequence appears in the Appendix. The construct had previously been shown to bind to Fas receptor and to cause cell death of Fas-expressing cells in a cytotoxicity assay (*11*).

3. Biolistic transfection.

Gold particles (BioRad) of 1.6 μm diameter were used as microprojectiles and were found to have good uniformity by scanning electron microscopy (Hitachi S-2500, see Chapter II). Particles (30 mg) were washed initially with 95% ethanol, twice with sterile water, then pelleted and resuspended in 500 μL of sterile water (final concentration of 60 mg/mL). Each disc was prepared using a separate DNA precipitation reaction, and the entire reaction mixture was loaded onto the macrocarrier disc. Based on our previous studies, we felt that we had to increase the total amount of DNA used for transfection. Therefore, three different quantitative combinations of each DNA and gold particles were used in the initial study: 3.0 μg DNA on 0.5 mg gold (our original conditions), 6.0 μg DNA on 0.5 mg gold, and 6.0 μg DNA on 1.0 mg of gold. Based on the results, in the subsequent transplantation studies, 6.0 μg of DNA on 1.0 mg of gold was used. The appropriate DNA, consisting either of the expression vector alone (no gene insert), or the expression vector containing the gene for human CTLA4-Ig or human soluble Fas ligand, was added to 16.6 μL of Au particles (60 mg/mL). In the case of the combination, a mixture of 3.0 μg each of the CTLA4-Ig and soluble Fas ligand preparations were used. The particles were resuspended and a volume of 2.5 M $CaCl_2$ equal to the total volume of DNA and Au particles, followed by 1 M spermidine (1/5 of the volume of $CaCl_2$ used) was quickly added and mixed well. The mixture was left to settle at room temperature for a minimum of 10 min, and then spun very briefly in a microcentrifuge. The pellet was washed with 70% ethanol, re-pelleted, washed with 95% ethanol, re-pelleted and resuspended in 25 µL of 95% ethanol. Using a micropipettor, the particle slurry was then sprayed onto the central area (~7.5 mm in diameter) of a Kapton macrocarrier disc which was 2.54 cm in diameter and 0.06 mm in thickness (BioRad) and allowed to dry in a desiccator. For exposure to the biolistic blast, following 48 h of culture as outlined above, 500 islets were suspended in 0.10 mL of media, and placed on the top of an inverted cell strainer with a mesh size of 40 µm (Falcon), the center of which had been pre-wet with 0.1 mL of media. The strainer and blasting apparatus was encased in a small vacuum chamber (a modification to a hand-held version of the BioRad biolistic apparatus) under 15" Hg of vacuum. Using this system, the target distance between the stopping screen and the suspended cells was 25 mm. Following the blast, the islets were resuspended in 2 mL of RPMI 1640 media supplemented with 12 mM HEPES, 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 20 µg/mL amphotericin B. The islets were then incubated at 37°C in 95% air/5% CO_2 for 48 h. Unblasted control groups simply had the media changed and were cultured in the same manner as the test groups.

4. Islet transplants.

Following a 48 h post-transfection period, 2 or 3 aliquots (a final total of approximately 400 islets) blasted with the same cDNA were pooled and transplanted beneath the kidney capsule of an alloxan-induced diabetic BALB/c recipient (85 mg/kg i.v. via penile vein injection). The cessation of graft survival was defined as blood glucose levels of 16.5 mM on three consecutive readings.

5. Insulin determinations.

Isolated islets were harvested into 1.0 mL of cold acid-alcohol (0.18 M HCl in 75% ethanol) and placed at 4°C for 48 h. The supernatant was removed and placed at -70°C.

The cellular pellet was further extracted at 4°C for 24 h with 0.5 mL of cold acid alcohol. The second supernatant was collected and added to the previously collected aliquot. Samples were stored at -70°C until assayed.

Approximately 15 days following the return of blood glucose levels to the diabetic range, the engrafted kidneys and pancreata were removed from the islet transplant recipients. The small portion of kidney containing the rejected graft was placed into 0.5 mL of cold acid alcohol, finely minced with scissors and extracted at 4°C for 48 h. The supernatant was removed, stored at -70°C, and a further extraction in another 0.5 mL of cold acid-alcohol was performed for 24 h. Supernatants were pooled and stored as outlined above. Insulin extraction on whole pancreata was performed in a similar fashion using 2.0 mL of cold acid-alcohol in each step (final volume of 4.0 mL). Samples were assayed using an insulin radioimmune assay kit (Linco Research Inc.).

6. ELISAs.

i) Human CTLA4-Ig

Round-bottomed 96-well plates (Nunc) were loaded with 50 µL/well of a 1/1000 dilution of AffiniPure goat anti-human IgG, Fcγ fragment specific antibody (Jackson ImmunoResearch Laboratories, Inc., 1.8 mg/mL) in 50 mM carbonate-bicarbonate buffer, pH 9.6 (Sigma), incubated for 30 min at 37°C, and stored at 4°C for no more than 3 days before use. Plates were blocked with 1% bovine serum albumin in PBS-Tween (1%) at 37°C for 1 h. Triplicate wells of 50 µL of either sample or standard (ChromPure Human IgG, Fc fragment, Jackson ImmunoResearch Laboratories, Inc.) diluted in RPMI were incubated for 1 h at 37°C. A 1/5000 dilution of alkaline phosphatase-conjugated AffiniPure goat anti-human IgG (Jackson, 0.6 mg/mL) in 1% PBS-T (50 µL) was added and incubated at 37°C for 1 h. For detection, 50 µL of pNPP (1.0 mg/mL in 0.1 M glycine buffer, 1.0 mM $MgCl_2$, 1.0 mM $ZnCl_2$, pH 10.4) was added per well, and the plate incubated for a minimum of 45 min at 37°C. The reaction was stopped with 50 µL of 3 N

NaOH per well. Plates were read on a Dynatech MRX microplate reader at a wavelength of 410 nm and a reference wavelength of 570 nm.

ii) Human Fas ligand

These assays were performed by Dr. Wei-ping Min in Dr Elliott's laboratory. Briefly, round-bottomed 96-well plates (Nunc) were coated with 50 μL of a 1.0 μg/mL solution of anti-human Fas ligand monoclonal antibody, kindly supplied by S. Nagata, Japan (*13*). Plates were incubated at 4°C overnight and then washed three times with PBS-Tween (0.1%). Sample or standard (prepared from the supernatant of COS cells transfected with the gene for soluble human Fas ligand) was added (50 μL) and incubated at room temperature for 1 h. Following another three washes with PBS-T, 50 μL of a 0.5 μg/mL solution of a second biotinylated anti-human Fas ligand monoclonal antibody (also supplied by S. Nagata) was added and incubation and washing carried out as before. Finally, 50 μL of a 1/4000 dilution of streptavidin-horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc.) was added and incubated for 45 min at room temperature. Colour was developed using TMB (Sigma Chemical Company, St. Louis, MO) and the reaction was stopped with 0.6 N H_2SO_4. Plates were read at 450 nm (test) and 570 nm (reference).

7. Histologic methods.

Following removal from the animal, engrafted kidneys or pancreata were placed in Bouin's fixative for 2 h, and then paraffin-embedded. Mounted sections underwent routine hematoxylin/eosin and aldehyde fuchsin staining. Immunohistochemical staining of paraffin sections for insulin was performed at room temperature as follows. Sections were quenched with 10% hydrogen peroxide/80% methanol for 6 min, blocked for 15 min with 20% horse serum, incubated for 30 min with a 1/1000 dilution of primary guinea pig anti-porcine insulin antibody (DAKO Corp., Carpinteria, CA), and for 20 min with a 1/200 secondary biotinylated goat anti-guinea pig IgG antibody (Vector Laboratories, Burlingame, CA). A Vectastain ABC kit (Vector Laboratories) and a liquid DAB Substrate Pack (BioGenex, San Ramon, CA) were used for color development, per the manufacturers' instructions.

RESULTS

Optimizing the amount of DNA and gold particles used for transfection

1. Assessing islet damage by insulin levels.

An initial study comparing the quantity of DNA and gold particles used for transfection was performed. When twice the DNA (6 μg) was used on the original amount of gold (0.5 mg), the particles became very clumped and difficult to resuspend following the DNA precipitation. As a result, the discs were very unevenly covered with large clumps of particles. For this reason that particular combination was not pursued. Use of 6 μg of DNA and 1.0 mg of gold particles overcame the clumping problem, and the islets subjectively appeared to contain many more gold particles microscopically than when 3.0 μg of DNA and 0.5 mg of gold were used.

We have used the ratio of the amount of insulin extracted from intact blasted islets to the amount of insulin released into the culture media following a 48 h post-transfection incubation as an indication of islet damage. The greater the amount of islet damage (with consequent insulin release into the surrounding media), the lower the ratio. Results appear in Table 5-1. Using our original conditions of 3 μg of DNA loaded onto 0.5 mg of gold, the range of the insulin ratios was 0.8 - 2.6 (mean value of 1.8). When twice the amount of both DNA and gold was used (i.e. 6.0 μg and 1.0 mg respectively), the range was 0.7 - 3.6 (mean value of 2.2). This difference was statistically significant ($p<0.05$, two factor

ANOVA), suggesting that there was somewhat less islet damage when both the amount of DNA and gold was doubled from that used previously. However, the mean insulin ratio for untreated islets was 5.1 (data not shown), again indicating that even with the better of the two conditions, there was still a considerable amount of islet damage (Chapter IV). There was also a statistically significant difference in ratios ($p<0.05$, two factor ANOVA) between the CTLA4-Ig-transfected and Fas ligand transfected groups, with the insulin ratios from the former group suggesting more islet damage.

2. Levels of transfected gene expression.

We measured the amount of CTLA4-Ig and Fas ligand secreted into the media during the 48 h incubation period which followed biolistic transfection of the islets. The ELISA results appear in Table 5-1. The lower limit of sensitivity was 75 pg/mL in both assays. In both DNA/gold groups, there was no detectable protein when islets were transfected with the empty vector alone. In the 3.0 µg DNA/0.5 mg gold group, there was no cross-reactivity of the molecules. However in the group with the larger amounts of DNA and gold, a small amount of cross-reactivity with Fas ligand was detected in the CTLA4-Ig assay. The most notable point from the data is the enormous variability in the amount of transfected gene expression within each subgroup (see particularly the CTLA4-Ig subgroup). Obviously, the blast to blast variability continues to be a major problem. As a result of this, it is difficult to interpret the differences in levels between the groups. It does not appear that there is a linear reduction in protein produced when half as much DNA is used (e.g. CTLA4-Ig levels with the DNA combination are not half of the levels with CTLA4-Ig DNA alone). Similarly, it does not appear that protein production is doubled when the amount of DNA/gold used for transfection is doubled; the levels appear to be similar. In as much as subjectively the islets appeared to contain more particles, the protein production appeared to be similar, and the amount of cell damage was less, all further studies were performed using a total of 6.0 µg of DNA and 1.0 mg of gold particles.

Transfection control grafts

We performed insulin extractions on pancreata from five BALB/c mice of a similar age to the recipients used in our study. Results appear in Table 5-2. The range of insulin values for the normal untreated pancreata was 24.8 - 65.5 ng/mg of pancreas (mean value of 40.9 ng/mg of pancreas). In contrast, at the time of nephrectomy, the pancreatic insulin levels were minimal in the alloxan-treated recipients with the exception of three animals. These animals were cases of alloxan reversion since there were minimal amounts of insulin in the engrafted kidneys. Mean values of 0.4, 0.8 and 1.1 ng/mg of pancreas were obtained for the islets cultured for 24 h, 72 h and the islets blasted with uncoated (naked) gold particles respectively, when the values from the reverted pancreata were excluded.

The syngeneic, blasted grafts survived until the time of nephrectomy (mean survival time of 66 days). In contrast, the mean survival time of the 24 h viability allograft controls was 17 days. The mean survival times of the 72 h cultured and naked gold-blasted islet allografts were slightly longer and almost identical (21 and 22 days respectively). Two weeks after their blood glucose levels had reached 16.5 mM, nephrectomies were performed on the mice containing the rejected allografts. Extraction of the engrafted kidneys for insulin revealed very minimal levels in all three groups - in the range of 0.001 - 0.005 μg of insulin with mean values of 0.002, 0.003 and 0.002 for the 24 h, 72 h culture and naked gold groups, respectively.

Transfected allogeneic islet transplants

1. Allograft survival

Table 5-3 shows the allograft survival times of the groups of islets which were transfected with the different DNA molecules. The two immunomodulatory molecules, CTLA4-Ig and Fas ligand, were inserted into different expression vectors, pPSC1 and pSCD7 respectively. All of the control grafts consisting of islets transfected with either of the empty vectors alone (i.e. no gene insert) were rejected. The mean survival time was 23 days which was very similar to the 72 h cultured, non-transfected group (21 days) and those blasted with naked gold (22 days, Table 5-2). In all of the control cases, minimal or undetectable insulin remained in the engrafted kidneys (mean of 0.002 μg) or the pancreata (mean of 1.3 ng/mg of pancreas). Again, these results were very similar to the values obtained for the naked gold particles (0.002 μg and 1.1 ng/mg of pancreas respectively, Table 5-2).

The length of graft survival of transfected islets is also shown in Table 5-3. Interestingly, there is a bimodal distribution in all three groups (compare with Chapter III). The grafts either rejected around the time of the empty vector controls (approximately 20 days) or survived longer term (>45 days). The cessation of graft survival was originally defined as blood glucose levels of 16.5 mM on three consecutive readings. Two weeks after reaching this point, the pancreas and engrafted kidney were removed for insulin extraction. However, if normoglycemia was maintained until 60 days, then the engrafted kidney was removed and sent for histology and blood glucose levels were monitored for the next 5 days to verify a return to hyperglycemic levels. At that time, the pancreas was removed and extracted for insulin. Disappointingly, as shown in Table 5-3, in all of the cases that went to 60 days, there was no return to the diabetic state following the nephrectomies (three cases in each DNA group). This indicates that the pancreata had regained their insulin-secreting capacity some time over the life of the graft. Indeed, the insulin contents of the pancreata from these cases were very high (range of 9.6 - 23.6 ng/mg of pancreas), providing confirmation of alloxan reversion. Unfortunately, because of the study design, insulin extraction was not done on the engrafted kidneys which would have indicated how much viable islet tissue was still present. However, upon re-examination of the blood glucose readings from these animals, it was evident that in all cases there had been a sudden increase in blood glucose levels from the baseline levels around 6 mM to somewhere between 10 and 12.5 mM. The elevated blood glucose levels remained within that range for approximately 3-5 days, and then returned to euglycemic levels (Figure 5-1). Interestingly, in all of these cases but one (#23), this spike in blood glucose occurred around 50 days, which is very similar to the time when the other long term grafts rejected. It is reasonable to assume that this peak corresponded to the time of graft rejection but that shortly thereafter, the insulin produced by the pancreas reached high enough levels to restore the blood glucose to the normal range. Taking this into consideration, 4 of 8 grafts (50%) of CTLA4-Ig-transfected islets survived >45 days (#10-13). Survival frequencies for this length of time in the other groups were 4 of 5 grafts (80%) in the Fas ligand group and 3 of 7 grafts (43%, # 23 classified as short term) in the combination of CTLA4-Ig and Fas ligand group.

One other point remains to be made in terms of allograft survival. As outlined above, this definition is an arbitrary one. We considered the graft to have failed when insufficient engrafted insulin-producing tissue remained to normalize blood glucose levels in a chemically-induced diabetic animal. It does not mean that the entire graft was destroyed and that there was no islet tissue remaining. Examination of the insulin levels from the kidneys containing rejected grafts confirmed this. In the CTLA4-Ig group, there was still a moderate amount of insulin-containing tissue in #8 (0.124 µg), although normoglycemia was not sustained beyond 23 days. In the case of #10, which rejected at 47 days, there was still a considerable amount of protected insulin-producing tissue remaining (0.75 µg of insulin compared to 0.002 µg in controls). Unfortunately, it was not enough to reach the critical level required to maintain euglycemia. Similarly, in the Fas ligand group, the graft which survived to 50 days (#15) actually contained 1.074 µg of insulin, well above the failed control grafts. In the DNA combination group, the graft which failed at 47 days (#22) contained a small amount of insulin (0.030 µg), while the one which failed at 18 days (#20) had a moderate insulin level (0.260 µg).

Finally, as previously mentioned, an untreated BALB/c pancreas from a mouse of similar age contained from 20 to 65 ng of insulin/mg of pancreatic tissue (Table 5-2). It is evident from the pancreatic insulin levels shown in Table 5-3, that levels were reasonably low at <21 days post-transplantation (0.4 - 1.7 ng/mg of pancreas). A value of 3.2 ng/mg of pancreas in a case at 25 days appears to be an exception. However, by 47 - 50 days, levels had increased to 2.1 (CTLA4-Ig group), 2.1 (Fas ligand group) and 4.9 ng/mg of pancreas (CTLA4-Ig/Fas ligand group). A rapid rise followed, since by 60 days the pancreatic levels had reached an average of 14.7 ng/mg of pancreas, enough to have returned the blood glucose levels to within the normal range.

2. Histology of kidneys harvested at 60 days.

Figures 5-2 through 5-4 are photomicrographs of the grafts which were harvested at 60 days. A summary of the histological appearance of the grafts appears in Table 5-4. Only one case (#13) in the CTLA4-Ig group shows a large well-granulated graft which is surrounded by mononuclear peri-infiltrates. Interestingly, this animal's jump in blood glucose levels at Day 50 was the least great, only reaching 10.4 mM with levels steadily declining back to normal over the following 5 days. In the other two long term CTLA4-Ig cases, no remaining islet tissue was seen in the sections examined, and the area of the grafts appeared relatively quiescent with little or no lymphocytic infiltration remaining. In the Fas ligand group, one case (#16) had some scattered islands of islet tissue remaining within a sea of mononuclear cells. The other two cases showed evidence of lymphocytic infiltration with no islet tissue remaining. In one case from the combination DNA group (#25), a few scattered islets were visible but most of the graft area was scar tissue with a moderate mononuclear cell infiltrate. The other case in this group showed very little evidence of active immune activity and was predominantly scar tissue.

Although all of these animals failed to return to the diabetic state following nephrectomy of the engrafted kidneys, and all had significant pancreatic insulin levels, no islets were seen in the pancreatic histological sections in half of them. This is due to the fact that only a small piece of pancreas was removed for histology since the rest was needed for insulin extraction. It emphasizes the point however, that a small number of histological sections may not be representative of the entire graft.

3. Relationship between allograft survival and pre-transplant transgene expression.

Table 5-5 shows the results of the ELISAs performed on the 48 h post-blast culture supernatants and the corresponding allograft survival times. Firstly, there are two aberrant false positive CTLA4-Ig values within the controls. They are probably the result of some cross-reactivity of the detecting antibody and a contaminant of some sort. The cases appear in order of increasing allograft survival. Unfortunately, it is evident that there is not a matching increase in levels of transgenic protein produced by the transfected islets. In other words, there is no correlation between the levels of protein produced within the pre-transplantation 48 h culture period and the length of allograft survival (correlation co-efficients of 0.159 for CTLA4-Ig group and 0.418 for Fas ligand group).

DISCUSSION

Optimizing the amount of DNA and gold particles used for transfection

In our earlier transplant study using CTLA4-Ig (Chapter III) we used 3.0 μg of DNA coated onto 0.5 mg of gold particles. We had concluded in that case that we were not reliably achieving an adequate amount of gene expression and subsequent protein production. We therefore felt that it was necessary to try to increase the amount of DNA used for transfection in this study. This would be of particular importance in the case of the combination of the two immunomodulatory molecules, where only 1.5 µg of each DNA would be used if the previous total of 3.0 µg was maintained. Having therefore decided to double the amount of DNA employed, the question then arose as to whether the previous or double the amount of gold particles should be used. We understood that the ratio of DNA to gold particles was critical and had been carefully determined previously during the development of the biolistic procedure (personal communication, Bill Heiser, BioRad). This was borne out by our experience. When double the amount of DNA was used with the lesser amount of gold, the particles became irreversibly clumped into large aggregates during the DNA precipitation procedure. In contrast, irreversible clumping was not a problem if the amount of both the DNA and gold were doubled. However, the quality and type of DNA preparation itself also affected the outcome of particle preparation. For example, it has been our experience that if the DNA was prepared using a Qiagen Maxi Kit (Qiagen Inc., Chatsworth, CA), the particles tended to clump much more than if the DNA was purified on a cesium chloride/ethidium bromide gradient. In this study, cesium chloride preparations were used for both CTLA4-Ig and Fas ligand. Nevertheless, the CTLA4-Ig coated particles were consistently slightly clumpier and more difficult to work with. This probably accounts for the slightly more damaging effect of the CTLA4-Ig blasts than that of the other DNAs (Table 5-1). It is not clear why blasting with twice as many particles is less damaging (Table 5-1). Perhaps a finer spray of particles is produced, or the helium shock is dissipated more under these conditions.

As previously mentioned, the ELISA data in Table 5-1 reveals how variable the transfection efficiency is between blasts. In several cases protein production is not even detectable, while within the CTLA4-Ig group alone there is a 1000-fold difference between only 4 blasts. In addition, the lower level of sensitivity for the assays is 75 pg/mL. In many of our cases expression was near this level. These factors make interpretation of the ELISA results difficult. It is not clear what effect using twice the amount of DNA is having in terms of protein production. The decision to use 6.0 µg of DNA and 1.0 mg of gold particles for the rest of the studies was therefore based on the microscopic appearance of the islets and the insulin results which suggested less islet damage under these conditions.

Transfection control grafts

The syngeneic islet allografts consisted of BALB/c islets which had been biolistically-transfected with a non-immunoregulatory reporter gene (either ß-galactosidase or firefly luciferase). The long-term survival of the syngeneic grafts (a minimum of 65 days until they were harvested) demonstrates that biolistically-transfected islets are capable of long-term in vivo function.

Unfortunately, because we had used all of our previous lot of collagenase, we had to use a different collagenase preparation for this study. Therefore, we included a viability control group which consisted of isolated allogeneic islets which had simply been cultured overnight prior to transplantation. Good graft function was obtained, and the mean survival time was 17 days. However, due to an increase in islet friability with the new collagenase, this in turn meant that for the transfected islet groups, we had to increase the post-isolation/pre-blast culture period from 24 h (used previously) to 48 h. Since long term culture has been used in the past as a means of immunomodulation, we therefore felt it was important to ascertain the allograft survival of islets which had not been transfected but simply cultured for the 72 h period. The range of allograft survival times in this case was 14 - 26 days with a mean of 21 days (excluding the cases of alloxan reversion which will be discussed in the next section). Therefore it would seem that the culture period alone has indeed shifted our baseline to a small extent. The slightly prolonged survival times are thought to be the result of two processes. Firstly, the islets have been hand-picked three times during the procedure which removes much of the more allogeneic exocrine tissue and ductal elements. Secondly, passenger leukocytes within the grafts do not tolerate in vitro culture as well as the endocrine tissue. The overall effect is a decrease in the immunogenicity of the tissue which shows up as a slightly longer time to rejection.

We also wanted to address whether the gold particles themselves had any immunosuppressive effect. The range and mean survival time of allografts blasted with gold which had not been coated with any DNA (naked) were very similar to the 72 h culture controls. Therefore, it is clear that the gold alone did not play any immunological role.

Finally, the amount of insulin extracted from the pancreas of a BALB/c mouse of similar age to our recipients was between 25 and 65 ng/mg of pancreas. Clearly, in our 24 h, 72 h cultured and naked gold blasted control groups, which were harvested approximately 35 days after transplantation, the pancreata have not regained significant insulin-producing activity (average of 0.8 ng/mg of pancreas). Similarly, there is negligible insulin extracted from the kidneys containing the rejected grafts in all control groups (0.001 - 0.005 μg of insulin).

Transfected allogeneic islet transplants

The results of the transplants using the empty vector controls (survival times, pancreas and engrafted kidney insulin contents) are very similar to the results of the 72 h culture and naked gold blasted controls. In other words, the presence of the empty DNA vector coated onto the gold particles does not have any effect.

The fact that the blood glucose levels did not return to the diabetic range in the grafts harvested at 60 days was disappointing to say the least. We have experienced similar problems in the past when using alloxan as a chemical inducer of diabetes. Its use is a double-edged sword: one wishes to determine maximal graft survival times, but it is well known that the incidence of the alloxan-treated pancreata regaining their insulin secreting capacity increases with time. It is interesting to note that, in general, the pancreatic insulin levels remain low in cases where the grafts are rejected in less than 26 days (72 h culture, naked gold, empty vector controls and transfected gene cases which had early rejection of their grafts - Tables 5-2 and 5-3). There are two exceptions; one animal in each of the 72 h culture and naked gold groups were reversions, which may have occurred early since hyperglycemia was never observed. However, in the animals which rejected their grafts around 45 - 50 days, the pancreatic insulin levels have increased approximately three-fold from earlier levels. Following this there must be a rapid rise since, by 60 days, the mean pancreatic insulin content is 14.7 ng/mg of pancreas, about one third of normal levels. This is obviously enough to normalize the blood sugar levels of the animals. We feel therefore, that in the animals which had their kidneys harvested at 60 days, there was a functional islet graft which maintained normal blood sugar levels out to at least 45 days. We interpret the presence of a sudden spike in the blood glucose levels to represent rejection of the graft. Figure 5-5 is a graphical representation of four hypothetical scenarios which could possibly occur with relation to changes in graft and pancreatic insulin levels, and which could explain the blood glucose readings obtained in the study. According to this proposed model the following possibilities could occur. In the case of the controls, there is a small amount of rapid graft loss, but the graft initially meets the required critical islet mass and blood glucose levels are normalized. As a result of continued immune attack, the graft is rejected by approximately 20 days. There has not been any pancreatic reversion and therefore, a critical islet mass no longer exists and blood glucose levels rapidly rise to the hyperglycemic range where they remain. In the case of an indefinitely surviving graft, the graft continues to provide the critical islet mass. There is no pancreatic reversion (e.g. see the 130 d and 164 d survivors in Chapter III) and steady blood glucose levels within the normal range are maintained. The third scenario occurs when there is partial destruction of the graft over time. Initially, the pancreas is not producing insulin and the islet graft functions to maintain euglycemia. By approximately 45 days the pancreas is beginning to regain its insulin-secreting capacity, but with the subsequent demise of at least some of the islet graft, a critical islet mass is not maintained. A window is therefore created around 45 - 50 days, where insufficient insulin to maintain normoglycemia is being produced, and blood glucose levels rise. Within the next few days, however, the pancreas rapidly regains its insulin-secreting capacity, the critical islet mass is re-established and blood glucose levels again return to normal. Similar events take place in the final situation, except that the islet graft is completely destroyed. In any event, a similar, short-lived window of elevated blood glucose levels is the result. It is not clear whether there is a relative difference in the final level or rate at which the pancreas regains its insulin-secretory function between the last two situations. Obviously the best way to closely follow the course of events would be to measure blood insulin levels. Unfortunately, this is not practical in a mouse model and we are left with the determination of blood sugar as an indirect reflection of insulin levels. While easy to perform, and relatively inexpensive, the blood glucose levels only provide us with information as to whether there is sufficient insulin being produced to maintain euglycemia, and do not reflect either the rate of decline in insulin levels produced by the graft or whether sub-optimal levels of insulin persist.

An interesting question is whether the presence of an islet graft has any influence on the incidence or kinetics of pancreatic reversion. It appears that the reversion process does not begin until after approximately 30 days. Once the reversion potential has been established, it is conceivable that the stress caused by the presence of hyperglycemia following demise of the graft speeds up the process of reversion which is why there is such a rapid increase in insulin production after the time of graft rejection. However, if the graft is rejected before 30 days, as in the control cases, the reversion potential has not been established and graft rejection results in hyperglycemia. An alternate possibility is that the islet graft itself produces some kind of factor which aids the reversion process at greater than 30 days. These questions could be answered in the first possibility by monitoring pancreatic reversion using a test system where exogenous insulin is provided continuously until 45 days versus 100 days. The use of syngeneic islet grafts which should survive indefinitely would address the second possibility.

The use of blood sugar measurements is a reflection of graft survival as long as the levels revert to the hyperglycemic range upon removal of the graft. Elevated blood glucose levels indicate when there is no longer the critical mass of insulin-producing tissue present which is required to normalize blood sugars. It is obvious from the insulin levels present in the "rejected" grafts that some functional tissue still remains. This point is also confirmed by the presence of insulin-producing tissue in some of the histological sections of the kidneys bearing "rejected" grafts. It is fair to say that some islet tissue in these cases has been protected and therefore escaped immune destruction; there is just not enough tissue remaining to maintain normal blood sugar levels. A more accurate reflection of graft cell survival would be obtained with either insulin or C-peptide levels, the problem being that there is no way of determining whether they are of graft or pancreatic origin unless the graft is xenogeneic and therefore produces a protein which is distinct from the host.

Just as a critical level of insulin is required to maintain euglycemia, it appears that a critical level of the immunomodulatory proteins is also necessary to provide graft protection. This is suggested by the fact that we see a bimodal response with the islets transfected with either of the immunomodulatory genes: the grafts either reject over the same time frame as the controls or they survive long term. No spectrum of survival times is seen.

There does not appear to be any correlation between the levels of protective protein produced during the 48 h incubation period following transfection and allograft survival (Table 5-5). There are several possible explanations for this. The protein in the culture supernatant includes that produced by cells which are part of an intact islet, as well as that produced by single cells. However, only intact islets are later transplanted. It may be that protein produced from single cells represents a significant proportion of the total, since the cells may have been knocked off of the periphery of an islet when they were hit by a particle (i.e. were transfected). Additionally, perhaps the initial level of gene expression does not reflect the subsequent in vivo situation. It is possible that the islets which produce more protein initially lose expression more quickly later on, whereas the ones which take longer to recover may show expression for longer. In the case of CTLA4-Ig, the number or position of the APCs within the islet may also make a difference . For example, the majority of protein secreted by peripherally located cells may end up in the media. Other cells, situated nearer an APC may not produce as much protein, but a larger proportion of it would become bound to B7 molecules. In the case of Fas ligand, the protein has to diffuse through the tissue to reach infiltrating mononuclear cells so the physical location of the cells producing the protein may be important.

We have demonstrated long term allograft survival (>45 days) of transfected islets in 50% of cases using CTLA4-Ig, both in our earlier and later study. This compares favorably with the results reported by Chahine et al. who achieved survival >51 days in 1 of 10 allografts using a co-transplantation model (*14*). As discussed earlier (Chapter III), the improvement could be due to the geographical location of the CTLA4-Ig production, as well as the fact that our islets (and their APCs) had been exposed to CTLA4-Ig prior to their transplantation.

Our success rate of prolonged allograft survival using Fas ligand-transfected islets was 80%. Unfortunately, we had a smaller number of final cases within this group due to some technical failures during the study (the islets in these isolations were more friable resulting in small transplant volumes and subsequent failure to correct hyperglycemia). However, our Fas ligand results agree with those obtained by Lau et al. (*15*) who achieved successful long-term protection in 100 % of allografts using a co-transplantation model of islets and 2 x 10^6 myoblasts which had been transfected with Fas ligand. The same authors show a spectrum of survival dates, with a direct relationship between the length of survival and the number of co-transplanted myoblasts (and presumably the amount of CTLA4-Ig produced). Unfortunately, nephrectomy was not performed on any of the long-term survivors in the above group; a definite cause for caution in interpretation of their results in light of our experience with spontaneous reversion of chemically-induced diabetic recipients.

One possible explanation for our inability to protect all grafts is that we do not achieve the minimal required level of protective protein in a number of cases due to the variability in our transfection efficiencies. This variability is clearly shown in Tables 5-1 and 5-5. Despite improvements to our methodology to increase transfection efficiency, we did not see any improvement in our success rate from that obtained previously with CTLA4-Ig (Chapter III). In some of the previous transplant studies, levels as high as 500 μg of protein were administered repeatedly intravenously (*16, 17*). Similarly, as mentioned above, Lau et al. found longer survival rates with larger amounts of protein production (*15*).

However, it is also possible that the 'failures' have a biological as opposed to technical cause. Although the direct antigen presentation pathway is thought to play a key role in allograft recognition (see Chapter 1), it is also likely that the indirect pathway also contributes. Antigens which are shed from the engrafted tissue may be taken up and presented by recipient antigen presenting cells in the regional lymph nodes. In this case, the transfected gene product is not present in the vicinity. This may be a partial explanation for the lower success rate seen with CTLA4-Ig vs. Fas ligand. With the former, T cells which had become activated in the lymph nodes may not be affected by the CTLA4-Ig which they encounter in the vicinity of the graft when they re-circulate. Indeed, studies have shown that the time of CTLA4-Ig is critical, having to be present within the first 2-3 days post-transplantation (*18, 19*). In the latter case, activated T cells should still be susceptible to the cytotoxic action of the Fas ligand in the graft vicinity, regardless of whether the activation occurred via the direct or indirect pathway.

Theoretically, the combination of the two molecules should achieve the best results since they should act in complementary fashion. Under optimal conditions, CTLA4-Ig should inhibit T cell activation through the direct pathway to a large extent, while Fas ligand should cause destruction of any cells which do become activated through either insufficient B7 blockade or by indirect antigen presentation in a distant location. In our case, long term survival was only achieved in 43% of the grafts transfected with the combination of CTLA4-Ig and Fas ligand. Unfortunately, in this group our expression of each of the transfected genes was very low, with CTLA4-Ig being non-detectable in practically all cases, and Fas ligand being produced at very low levels in all cases but one. If a critical level of each molecule is required, perhaps the same amount of each DNA as was used in the single molecule studies (i.e. twice the total amount) should have been used. We were hesitant to attempt this since the ratio of DNA to gold particles seems to be critical. Alternatively, perhaps half of the islets should have been transfected with one molecule in higher concentration and the other half with the other molecule.

The quality of the collagenase preparation also contributed to a lower success rate in this study, since the islets were more fragile and susceptible to damage from the blast.

Despite the problems encountered with islet fragility, variability in transfection efficiency, insufficient gene expression and pancreatic alloxan reversion, we can conclude that both CTLA4-Ig and Fas ligand appear to be effective as locally produced immunosuppressive agents to achieve allograft protection. The hurdles which remain are to achieve more consistent transfection efficiencies and subsequent protein production, higher levels of protein production of each molecule when used in combination. This would allow better delineation of biological vs. technical limitations.

Table 5-1. The effect of the quantity of DNA and gold particles on islet damage and transgene expression.

DNA	Insulin ratio (islets/media)	ELISA (pg/mL) CTLA4-Ig	ELISA (pg/mL) Fas lig	Insulin ratio (islets/media)	ELISA (pg/mL) CTLA4-Ig	ELISA (pg/mL) Fas lig	Group Mean
	3.0 µg DNA/0.5 mg gold			6.0 µg DNA/1.0 mg gold			
Vector alone	1.8	ND	ND	1.6	ND	ND	2.0
	1.5	ND	ND	1.7	ND	ND	
	2.3	ND	ND	3.6	ND	ND	
	1.5	ND	ND	1.6	ND	ND	
Mean	1.8			2.1			
CTLA4-Ig	0.9	76	ND	1.0	109	ND	1.4 [a]
	0.8	132	ND	0.7	77	ND	
	2.2	1226	ND	2.3	741	ND	
	1.6	7749	ND	1.4	7978	ND	
Mean	1.4			1.4			
Fas ligand	2.1	ND	326	2.7	85	ND	2.5 [a]
	2.6	ND	ND	3.2	ND	536	
	1.8	ND	512	3.6	121	367	
	1.5	ND	600	2.1	ND	283	
Mean	2.0			2.9			
CTLA4-Ig/ Fas ligand	2.3	165	333	2.4	440	407	2.1
	1.1	440	424	1.9	ND	ND	
	1.8	554	431	2.8	154	ND	
	1.8	229	317	2.6	337	561	
Mean	1.8			2.4			
Group Mean	1.8			2.2 [b]			

[a] $p<0.05$, two factor ANOVA, followed by Tukey's post-hoc analysis

[b] $p<0.05$, two factor ANOVA

Table 5-2. Graft survival and insulin levels of transfection control grafts.

Group (n)	Weight (mg)	Total insulin (µg)	Insulin (ng/mg panc)	Total Insulin (µg)	Graft Survival (days)
	Pancreata			Engrafted Kidney	
Untreated mice (n=5)	463	11.47	24.8		
	475	13.18	27.8		
	380	12.79	33.7		
	535	28.34	53.0		
	350	22.93	65.5		
Mean		17.74	40.9		
Syngeneic grafts * (n=4)					66
					66
					65
					65
Mean					66
24 h viability controls (n=6)	246	0.15	0.6	0.003	17
	310	0.09	0.3	0.003	15
	277	0.10	0.3	0.003	19
	299	0.09	0.3	0.002	20
	281	0.10	0.4	0.002	15
	471	[4.8]	[10.1]	0.001	[60]
Mean		0.10	0.4	0.002	17
72 h culture pre-tx (n=7)	149	0.15	1.0	0.002	26
	192	0.17	0.9	0.003	14
	221	0.16	0.7	0.004	21
	235	0.14	0.6	0.005	19
	226	0.18	0.8	0.002	22
	296	0.27	0.9	0.002	26
	367	[5.3]	[14.4]	0.002	[60]
Mean		0.18	0.8	0.003	21
Blasted with naked gold pre-tx (n=6)	243	0.18	0.8	0.003	23
	212	0.26	1.2	0.003	23
	289	0.16	0.6	0.003	18
	322	0.38	1.2	0.002	23
	394	0.66	1.7	0.001	23
	451	[5.00]	[11.0]	0.001	[60]
Mean		0.33	1.1	0.002	22

All recipients in transplant groups were alloxan-diabetic.
Values in brackets represent alloxan reversion and are not included in the calculation of the mean.
* Nephrectomy performed at this time. Blood glucose levels returned to the hyperglycemic range in all cases.

Table 5-3. Graft survival and insulin extractions of transfected islet transplants.

Group	#	Graft Survival (days)	BG Spike[a] (day)	Wt (mg)	Total Insulin (µg)	Insulin (ng/mg panc)	Total Insulin (µg)
				Pancreas			Engrafted Kidney
pPSC1[b]	1	20		319	0.38	1.2	ND
(n=2)	2	29		317	0.62	2.0	0.004
pCSD7[c]	3	15		300	0.23	0.8	ND
(n=3)	4	21		292	0.50	1.7	0.002
	5	30		291	0.30	1.0	0.002
Mean		23			0.41	1.3	0.002
CTLA4-Ig	6	15		325	0.33	1.0	ND
(n=8)	7	18		319	0.49	1.5	0.002
	8	21		350	0.13	0.4	0.124
	9	25		348	1.10	3.2	ND
	10	47		256	0.54	2.1	0.750
	11	60[d]	46	345	6.43	18.6	NA
	12	60[d]	47	390	6.85	17.6	NA
	13	60[d]	50	426	4.85	11.4	NA
Fas Ligand *	14	23		292	0.19	0.6	ND
(n=5)	15	50		454	0.98	2.1	1.074
	16	60[d]	50	336	3.23	9.6	NA
	17	60[d]	53	389	6.22	16.0	NA
	18	60[d]	55	323	4.44	13.8	NA
CTLA4-Ig/	19	17		360	0.34	0.9	ND
Fas Ligand	20	18		332	0.24	0.7	0.260
(n=7)	21	20		355	0.16	0.5	0.002
	22	47		451	2.20	4.9	0.030
	23	60[d]	23	362	4.02	11.1	NA
	24	60[d]	48	341	8.05	23.6	NA
	25	60[d]	55	317	3.23	10.2	NA

ND = non-detectable
NA = not available

[a] Day when blood glucose suddenly rose from baseline.
Levels failed to reach 16.5 mM and returned to normoglycemic range.

[b] Empty vector used with CTLA4-Ig

[c] Empty vector used with Fas ligand

[d] Blood glucose levels did not return to diabetic range following nephrectomy of engrafted kidney.

* $p<0.05$, Fisher's Exact Test for survival >45 days.

Table 5-4. Histological appearance of long term allografts harvested at 60 days.

Group	#	BG Spike* (days)	Histological Appearance Kidney	Pancreas
CTLA4-Ig	11	46	No graft visible. No lymphocytic infiltrate, scarring.	No islets seen
	12	47	No graft visible. Small lymphocytic infiltrate.	Occasional islet seen.
	13	50	Large well-granulated graft. Large lymphocytic peri-infiltrate.	No islets seen.
Fas ligand	16	50	Scattered islands of graft. Large lymphocytic infiltrate.	Many islets seen.
	17	53	No graft visible. Moderate infiltrate.	No islets seen.
	18	55	No graft visible. Moderate infiltrate and scarring.	Occasional islets visible.
CTLA4-Ig/ Fas ligand	24	48	No islets seen. Scarring, very little infiltrate.	No islets seen.
	25	55	Few scattered islets seen. Moderate lymphocytic infiltrate.	Several islets seen.

* Day when blood glucose suddenly rose from baseline.
Levels failed to reach 16.5 mM and returned to normoglycemic range.

Table 5-5. Relationship between allograft survival and pre-transplant transgene expression.

Group	Case #	Survival (days)	BG Spike (days)	ELISA (pg/mL)	
				CTLA4-Ig	Fas Ligand
pPSC1	1	20		413	ND
	2	29		ND	ND
pCSD7	3	15		ND	ND
	4	21		ND	ND
	5	30		ND	ND
CTLA4-Ig [a]	6	15		326	ND
	7	18		325	ND
	8	21		428	ND
	9	25		148	ND
	10	47		229	ND
	11	60	46	1020	ND
	12	60	47	157	ND
	13	60	50	118	ND
Fas Ligand [b]	14	23		ND	190
	15	50		ND	326
	16	60	50	ND	454
	17	60	53	204	145
	18	60	55	ND	331
CTLA4-Ig/ Fas Ligand	19	17		ND	180
	20	18		ND	426
	21	20		90	ND
	23	23*		ND	114
	22	47		82	180
	24	60	48	ND	ND
	25	60	55	ND	ND

ND = non-detectable
NA = not available
[a] correlation co-efficient = 0.159
[b] correlation co-efficient = 0.418
* Assumed to have rejected at 23 d.

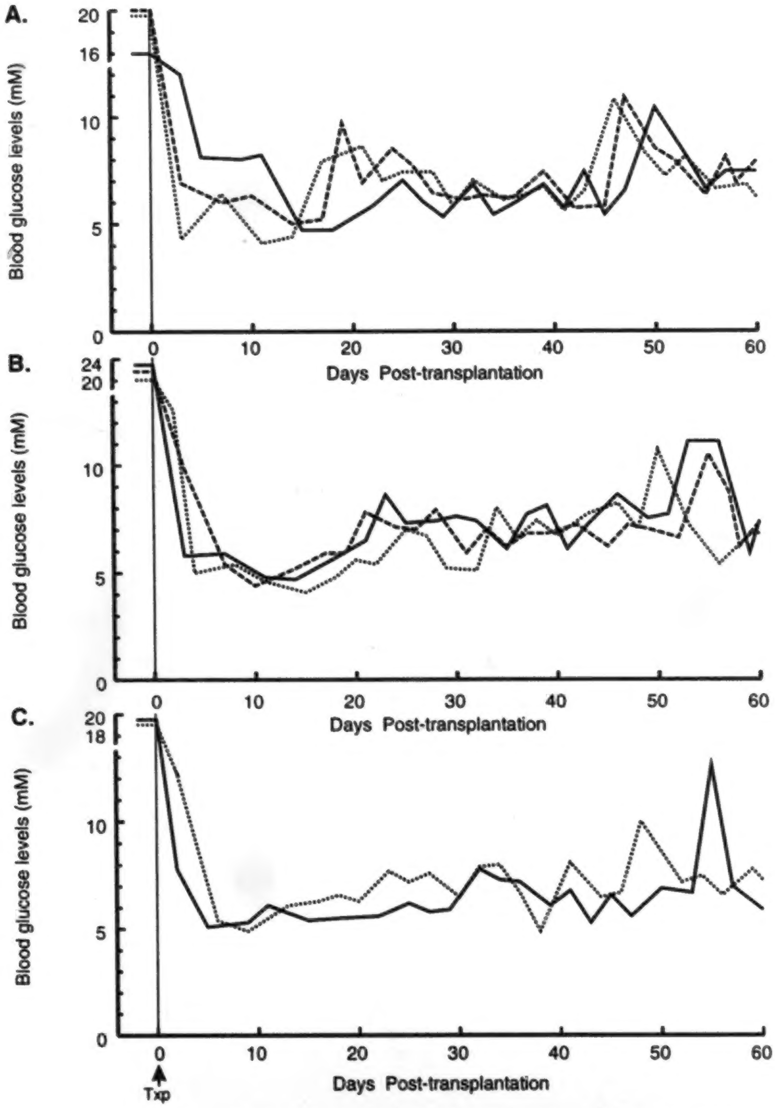

Figure 5-1. **Blood glucose levels of mice bearing transfected long-term allografts, harvested at 60 days.**

A. Diabetic BALB/c recipients who received CBA islets transfected with the gene encoding human CTLA4-Ig.

B. Diabetic BALB/c recipients who received CBA islets transfected with the gene encoding human soluble Fas ligand.

C. Diabetic BALB/c recipients who received CBA islets transfected with the genes encoding human CTLA4-Ig and human soluble Fas ligand.

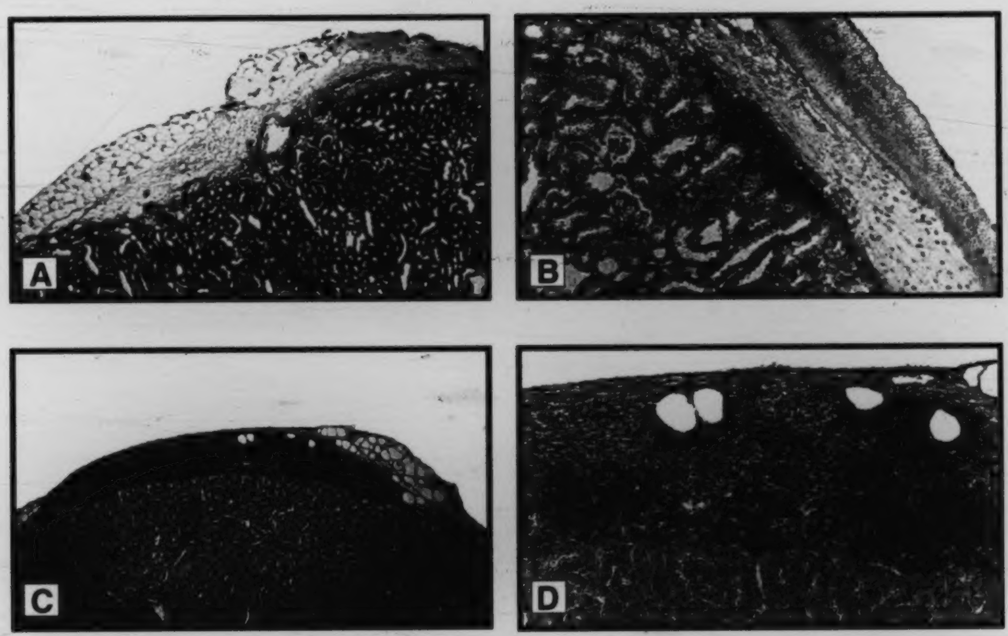

Figure 5-2. **Photomicrographs of histological sections from kidneys harvested at 60 days which were transplanted with allogeneic islets transfected with human CTLA4-Ig.**

A. H + E stain of Sample #11, blood glucose spike at 46 days.

B. H + E stain of Sample #12, blood glucose spike at 47 days.

C. Insulin immunohistochemical stain of Sample #13, blood glucose spike at 50 days.

D. High power view of insulin immunohistochemical stain of Sample #13.

See Table 5-4 for interpretation.

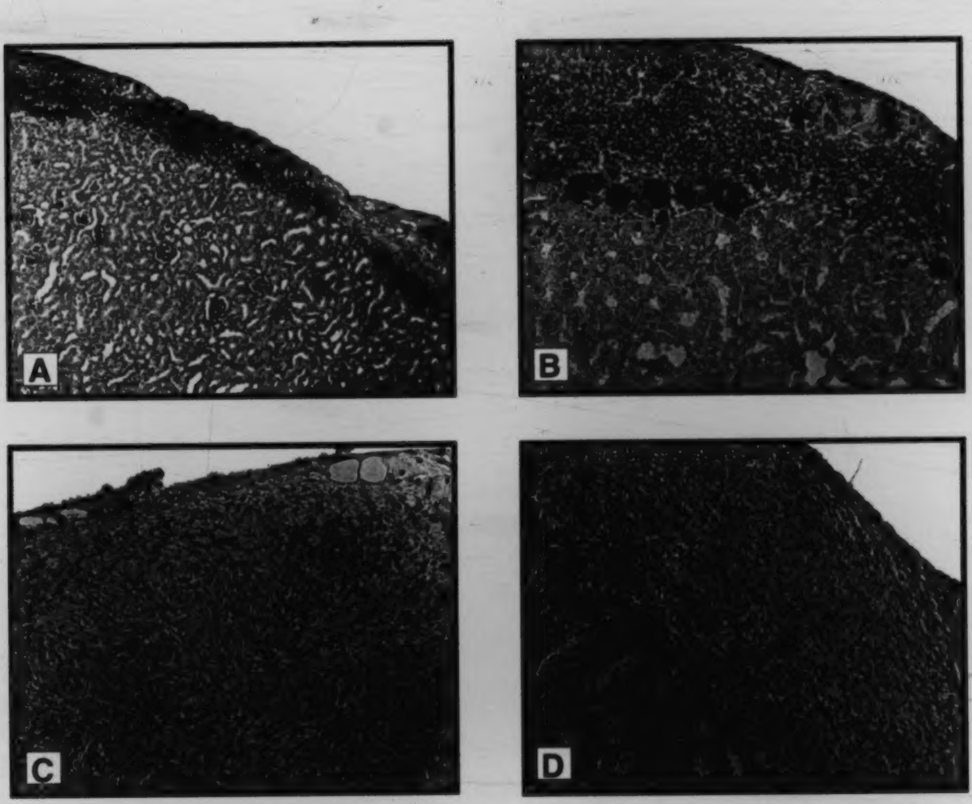

Figure 5-3. Photomicrographs of histological sections from kidneys harvested at 60 days which were transplanted with allogeneic islets transfected with soluble human Fas ligand.

A. Insulin immunohistochemical stain of Sample #16, blood glucose spike at 50 days.

B. High power view of insulin immunohistochemical stain of Sample #16.

C. H + E stain of Sample #17, blood glucose spike at 53 days.

D. H + E stain of Sample #18, blood glucose spike at 55 days.

See Table 5-4 for interpretation.

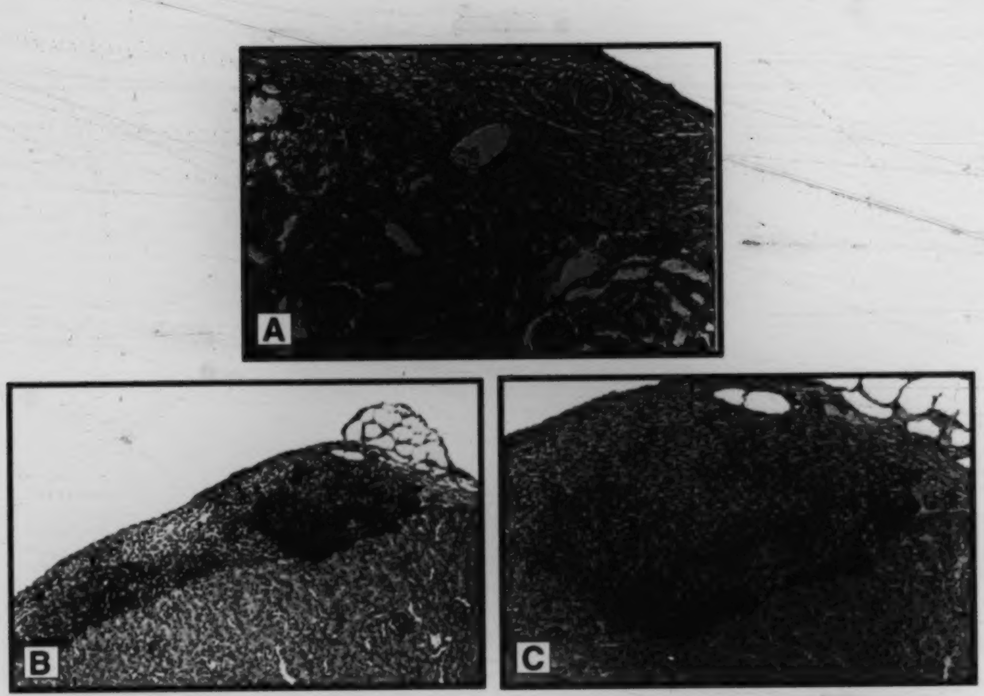

Figure 5-4. **Photomicrographs of histological sections from kidneys harvested at 60 days which were transplanted with allogeneic islets transfected with a combination of human CTLA4-Ig and human soluble Fas ligand.**

A. H + E stain of Sample #24, blood glucose spike at 48 days.

B. Insulin immunohistochemical stain of Sample #25, blood glucose spike at 55 days.

C. High power view of insulin immunohistochemical stain of Sample #25.

See Table 5-4 for interpretation.

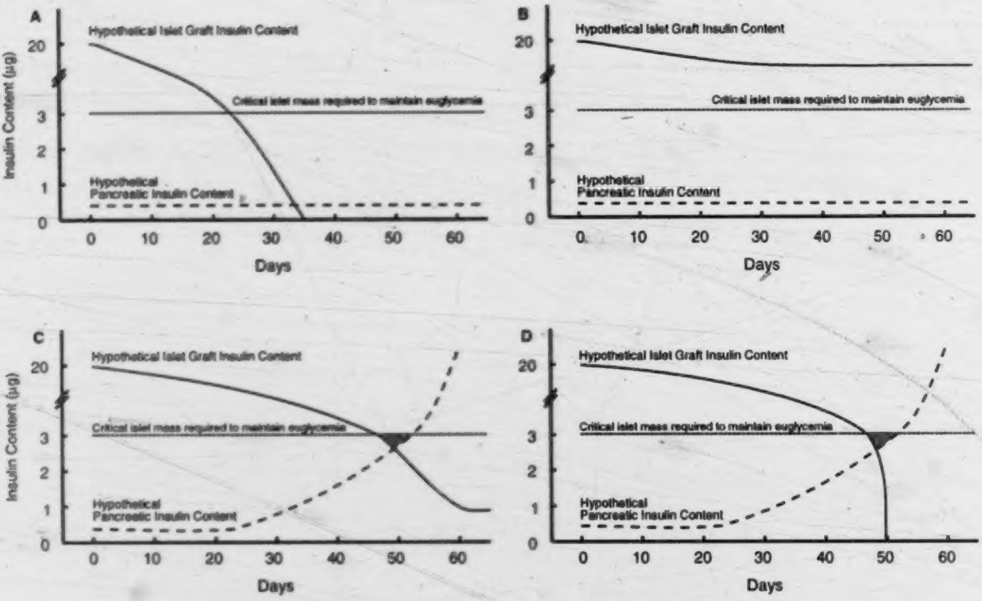

Figure 5-5. **Hypothetical scenarios of allograft and pancreatic insulin content in alloxan-induced diabetic mice.**

A. Rapidly rejected islet graft and no pancreatic reversion.

B. Indefinitely surviving islet graft and no pancreatic reversion.

C. Longterm survival of partial islet graft and pancreatic reversion.

D. Complete rejection of islet graft after prolonged survival and pancreatic reversion.

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CHAPTER VI

GENERAL DISCUSSION

The appeal of successful islet transplantation as a treatment for Type I diabetes is obvious: it is the most physiologic method of insulin replacement with independence from multiple daily insulin injections, perhaps the most important consideration from a patient's point of view. Exogenous insulin independence would have enormous implications in terms of quality of life, and a return to a continuous euglycemic state would prevent any (further) disease complications. In essence, the disease would be cured. In order for this treatment modality to become a widespread reality for juvenile diabetics, however, we must be able to achieve long-term survival and function of the islet graft in the absence of chronically administered immunosuppressive drugs. This entails overcoming the problems of primary non-function of the graft as well as both the alloimmune and autoimmune rejection processes.

The intent of this study was to explore the feasibility of transfecting islets with genes for immunomodulating molecules before transplanting them as a source of insulin-producing tissue, in the hopes of preventing allograft rejection. The appeal of this gene therapy approach is that it would allow localized immunosuppression, limited to the area of the graft. Chronic administration of oral pharmaceutical agents, their toxic side effects and the resultant susceptibility to particular pathogens could therefore be avoided. Direct transfection of the islets would achieve two purposes. Firstly, it would eliminate the need for a second type of tissue that would have to be immunocompatible, transfectable and non-mobile (so that it remained in the area of the engrafted islets). Secondly, toxic side effects of the immunoregulatory molecules themselves could be minimized.

The two main questions to be addressed were: 1) How could intact islets be transfected? 2) What genes could be tried to achieve the desired effects? We used an allogeneic mouse model consisting of isolated CBA (H-2^k) islets transplanted into BALB/c (H-2^d) recipients to explore the answers to these questions.

BIOLISTIC TRANSFECTION OF INTACT ISLETS

Although several studies have shown that lipofection (*1, 2*) and electroporation (*1, 3-5*) can efficiently transfect dispersed islet cells, there is only one study that reports successful transfection by lipofection using intact islets (*6*). In the present study, transfection using Lipofectamine (GIBCO/BRL) on intact mouse islets was unsuccessful (*7*). As discussed in Chapter I, this transfection inefficiency is probably due to a combination of the overall architecture of an islet and the presence of DNA-degrading acid proteases in the lysosomes of the islet cells. However, it was felt that further pursuit of a non-viral transfection method was worthwhile for a number of reasons. Gene therapy which utilizes adenoviral vectors has been limited by the fact that infection of adult immunocompetent hosts produces only transient recombinent gene expression in vivo. Due to the immune responses which are produced, it has thus far not been possible to successfully readminister virus of the same serotype (*8-13*). Both cellular and humoral imnmune responses are responsible for both the transient gene expression and for the inability to readminister virus following an initial infection (*14-16*). In the case of currently available retroviruses, the risk of oncogenesis limits their widespread applicability. Biolistics seemed an obvious choice to pursue since it had been used to successfully transfect a variety of primary tissues which were previously found to be either difficult or impossible to transfect by more widely-used techniques (*17, 18*). Due to the nature of the biolistic transfection process, neither the islet architecture or the lysosomal contents present a problem since the naked DNA is physically forced into the cytoplasm of the cells. The studies presented in Chapter II have clearly shown that biolistics is a feasible transfection method for intact mouse islets (*7*). Furthermore, it was verified that the surviving transfected islets maintained their insulin secretory capacity to glucose both in vitro and in vivo.

1) Advantages

Perhaps the greatest point in favour of biolistics is that it is a non-viral transfection method. The benefits are twofold. The first benefit is the lack of an immune response to the gold particles used as the DNA carrier. Although currently available adenoviral transfection methods have been shown to be very efficient on islets (*19-21*), we have reservations about their use in transplantation due to the immune responses mounted against the viral proteins (*22*). We approached these studies with the ultimate goal of human islet transplantation in mind. Since the islets themselves are the transfection targets, viral transfection and subsequent viral protein production could result in immune clearance of the islet graft. It could be argued that this point shouldn't be a concern since our approach should block anti-viral as well as anti-graft immune responses. However, it is doubtful that we would be successful in blocking all responses, and it would provide additional target antigens to the system. Even if the graft was not destroyed, it seems counterproductive to invoke any kind of immune response, when the actual goal of the procedure is to dampen the immune response to the transplanted tissue. The advantage of using gold particles as the DNA microcarrier is that gold is a chemically inert substance. Further, gold has long been approved for use in humans, most often being employed in patients with auto-inflammatory conditions. In these cases, there is no inflammatory response to the gold. Indeed, in our mouse studies, there is no difference in the mean survival times between untreated islets and those which have been blasted with 'naked' gold particles (Chapter V).

The second benefit of biolistics is that once the gene is cloned, no further lengthy preparation time is required. Recombinant viruses are labour-intensive and can take months to prepare. Further, a viable product can sometimes be difficult to obtain. Indeed, our initial attempts to prepare a CTLA4-Ig-producing adenovirus were time-consuming and unsuccessful. Despite the wealth of supporting literature in this area, there are still no reports of studies employing a CTLA4-Ig-expressing adenovirus. There are two major problems that can be encountered during recombinant adenoviral preparation: 1) The product is toxic to the cells. 2) The size of the virus exceeds the limits of packaging or the insert is unstable. Unfortunately, these problems only become apparent after considerable time and effort has been wasted. In contrast, biolistics provides a means to quickly screen molecules in terms of their therapeutic potential. Although it may not be the transfection method ultimately used (see Limitations below), perhaps only molecules which appeared to show some potential on an initial screen would be further pursued with lengthy and costly procedures.

2) Limitations

Despite the advantages outlined above, there were significant drawbacks to using biolistics on intact islets. First, we encountered significant islet/cell loss from the exposure to the biolistic blast. The islet recovery (number of islets remaining following the blast) ranged anywhere from ~35% to 70%. This varied with the helium pressure, gold particle size, and method of sample exposure used (hanging drop versus sitting drop). The factor noted to have the greatest impact however, was the state of the islets before exposure to the biolistic blast. The presence of a microscopically visible capsule surrounding the islet appeared to be critical. This in turn was influenced by the type and lot number of collagenase used to perform the digestion. For example, compared to the previous studies in Chapters II, III and IV, the studies performed in Chapter V required that the islets be cultured for an additional 24 h prior to exposure to the blast, in order to regain a robust capsule. Blasts done before this additional culture period resulted in total disruption of the islets. This was because a different lot of collagenase had to be used for the digestions in the later study, all of the previous lot having been consumed. Again, the lack of availability of large amounts of a reproducible, effective collagenase continues to be an ongoing frustration within the realm of islet isolation.

A second limitation of the biolistic method for islet transfection is the low transfection efficiency achieved. Approximately 3 - 5% of the total cells within an islet were originally found to be transfected (Chapter II). Although the levels of transfected gene expression were later improved from this by about a factor of 18 (Chapter IV), we know that the accumulated total transgenic protein produced over a 48 h period was only in the order of picograms (Chapter V).

Related to the transfection efficiency is the problem of considerable variability in the amount of protein (encoded by the transfected gene) which was achieved from one blast to another. Within all of the groups of blasts performed, including both the in vitro and in vivo studies, there was often a factor of 25 to 30 between the highest and lowest values of reporter product obtained (a factor of 1000 in the CTLA4-Ig group in Chapter V). This occurred despite careful attention to performing the procedure as consistently as possible. The problem seemed to be inherent to the technology used, as considerable differences were seen in the pattern of tearing of the rupture discs, and the angle at which the macrocarrier discs hit the stopping screen when repetitive blasts were performed (Chapter IV). This variability may be, in part, responsible for the partial success rates in our gene therapy trials (Chapters III and V). Perhaps we are only achieving sufficient protein levels to provide protection in some cases, but in others the level of gene expression is simply too low.

Finally, as outlined in the introductory chapter, biolistics only achieves transient gene expression as the DNA remains in an episomal form. In our system, gene expression was found to decline steadily, with levels at 4 weeks post-transfection being 1/8 of those at

1 week post-transfection (Chapter II). Whilst transient expression may be sufficient in some instances, successful gene therapy in many cases would require ongoing expression of the transfected gene.

GENE THERAPY AND ISLET TRANSPLANTATION

1. Human CTLA4-Ig.

Our studies have shown long-term allograft survival in 50% of cases transfected with CTLA4-Ig (Chapters III and V). Interestingly, in general it appears that the grafts either survive long term or they reject around the time of the controls i.e. the process either works or it doesn't; we do not see a spectrum of intermediate survival times. This would seem to suggest that there is a minimum level of protein required in order to achieve the protective effect. Apart from the blast to blast variability in transfection efficiency noted above, there are a number of other possible explanations for why only a proportion of our cases attained long term survival.

We synthesized human CTLA4-Ig since it had previously been shown to be effective in mice (*23-25*), and because the ultimate aim is to explore this approach within the context of human islet transplantation. More recently murine CTLA4-Ig has been cloned (*26, 27*). Although the mechanisms of action are the same, the murine CTLA4-Ig molecule has been found to have approximately ten times higher binding affinity to the murine B7 molecule than the human counterpart (*26*). Perhaps if murine CTLA4-Ig had been used in our studies, the greater affinity between the two molecules would have resulted in more effective blockade of lymphocytic activation and a greater success rate in long-term allograft survival. Steurer et al. argue compellingly that the higher binding efficiency of the murine molecule is responsible for their improved islet allograft survival rates over other groups (*27*). However, it is possible that a molecule with too high an affinity for B7 may in fact be detrimental. Since it is now believed that B7 binding of CTLA4 on activated T cells plays a role in down-regulation, or containment, of the immune response (*28*), high affinity binding of CTLA4-Ig to the B7 which results in long-term blockade could in fact result in a de-regulated immune response.

Within the literature, it is very difficult to make comparisons between the in vivo CTLA4-Ig studies, due to the differences in routes of protein administration, dosing schedules, types of recipient animals, species from which DNA was cloned, etc. Early in vitro studies showed >75 % inhibition of murine T cell proliferation when concentrations of 0.1 - 1.0 μg/mL of human CTLA4-Ig were used (*29*). Later studies increased the levels from 1.0 - 10.0 μg/mL, but found 50 % inhibition using 10.0 ng/mL (*30*). However, in these cases the amount of stimulating and responder cells were in the neighbourhood of 3×10^5 lymphocytes. Our ELISA results indicate that anywhere between 80 and 1020 pg/mL of human CTLA4-Ig were produced by the biolistically-transfected islets within 48 h of culture post-transfection. However, for reasons discussed previously (Chapter V), it is difficult to know how this number relates to the amount of protein which is produced at the time of, or following transplantation. Similarly, in vivo studies which have employed human CTLA4-Ig protein have injected 50 μg q2d x 14 d (*23, 24*), 75 μg qd x 3 d (*31*), 100 μg on Day 0 and Day 1 of transplantation (*25*), 500 μg i.p. on Day 0 of transplantation alone, or x 7 d (*32*), and 500 μg on Day 0 and Day 2 of transplantation (*30*). Considering that the CTLA4-Ig molecule has a biphasic clearance with $T_{1/2}$ of approximately 4 h and 30 h, and that clearance of higher doses is more complex and dose-related (*31*), these values are again very difficult to relate to our situation.

Perhaps more relevant to our situation, are the co-transplantation studies which also result in localized expression of the transfected gene. Chahine et al. reported that 10^5 cells of the clone which they co-transplanted produced 70 ng/mL of CTLA4-Ig over a 36 h period (*33*). This is several hundred times more than we detected in our islet system

(Chapter V). However, despite this high in vitro production, only 3 of 7 recipients of the cells had any detectable serum levels of CTLA4-Ig. In the co-transplantation studies which utilized Fas ligand-expressing myoblasts, protein production was not quantified, but was instead detected by fluorescence-activated cell sorting (*34*).

Another concern is that with binding of the CTLA4-Ig to APCs, the Fcγ1 tail would bind complement and the APC would undergo lysis. While this would perhaps result in a decrease in overall immunogenicity, the achievement of anergy would no longer be a possibility (since a strong Class II Signal 1 in the absence of Signal 2 would not be present). Further, with the mutations introduced into the hinge area of the Ig tail of our molecule, the two heavy chain molecules are no longer cross-linked, and the complement binding sites are most likely destabilized (personal communication, Peter Linsley).

There is also the possibility that an immune response may be raised by the mouse to the human CTLA4-Ig protein and that the protein would be cleared from the system. Even if this were to be the case, it is unlikely to play a major role in this setting since we know that the transfected gene expression is limited in duration through the nature of the biolistic process.

A final problem also relates to the transient nature of the transgene expression. Despite initial graft protection , there is still the possibility of new thymic emigrants directed against the graft being released after the expression of the transfected gene has ceased. This could result in graft rejection further down the road.

2. Human soluble Fas ligand.

In our studies we achieved allograft survival >45 days in 80% of our cases. Although this was a somewhat smaller sample group than the others, it is in agreement with results obtained by Lau et al. who used a co-transplantation model (*34*).

Similar arguments to those used with human CTLA4-Ig pertain to the use of human Fas ligand in a murine model. We employed a human Fas ligand molecule to bind to murine Fas receptors on activated lymphocytes. Therefore, the problems of human-specific immune responses and differing binding constants may also apply.

Theoretically, the Fas ligand could require much higher levels of secreted protein than the CTLA4-Ig system for a number of reasons. In both systems, it is assumed that a certain critical level of protein is required for binding. The distance between the protein-producing islet cell and the target cell to be bound therefore becomes important. Due to loss through diffusion and dilution, more protein would be required for distantly located infiltrating lymphocytes (Fas ligand system) than for proximally located intra-islet APCs (CTLA4-Ig system). Additionally, 2 or 3 Ia-positive cells (APCs) were found within canine islets (*35*). In the CTLA4-Ig system, the amount of protein required is therefore limited to that needed to bind all of the B7 sites on this limited number of cells. Conversely, Fas ligand's mode of action is to bind to (and cause destruction of) activated T cells. The numbers of activated, infiltrating lymphocytes would be considerable, requiring relatively large amounts of protein to bind to them all.

Having said this, our studies do not show more effective results with CTLA4-Ig than with Fas ligand. This would seem to suggest that the quantity of the protein which is present is not the only important factor. Other mechanisms, such as indirect antigen presentation by recipient APCs in distant locations may also play a role (as discussed in Chapter V). In theory, it seems that a combination of the two molecules should be most effective: In the best of scenarios, T cell activation should largely be prevented by the CTLA4-Ig, but any cells which do in fact become activated could then be destroyed by apoptosis following binding of Fas ligand. Unfortunately, our results have not shown this to be the case. By using half as much of each of the cDNAs in order to keep the total amount constant, we achieved very little protein production of either molecule as reflected by our ELISA results.

ACHIEVEMENTS OF THIS STUDY

The achievements of this study are severalfold. This data is unique, in first exploring the concept of biolistic transfection of intact islets. Presentation of our data at the International Pancreas and Islet Transplantation Association's meeting in Miami, in 1995 generated considerable interest. While several other groups are now pursuing the concept we are still the only ones to date who have published on the subject. It would be fair to say that we are regarded as one of the leaders in the field of biolistic islet transfection.

Along similar lines, due to our development of this transfection technique, our data is unique in first demonstrating results of an in vivo trial using islets directly transfected with immunomodulatory molecules (CTLA4-Ig). While our long-term allograft survival success was only partial, we feel that we have demonstrated 'proof of principle' in terms of usefulness of both CTLA4-Ig and Fas ligand in prevention of allograft rejection. Indeed, our results confirm, in principle, earlier findings by several groups that CTLA4-Ig prolongs allograft survival. We have achieved the highest rates of long-term allograft survival to date with local production of CTLA4-Ig, with considerable improvement over the results shown Chahine et al. (*33*). In the case of soluble Fas ligand, our findings were similar to those of Lau et al. (*34*). Both of these groups were using co-transplant models of islet transplantation. The findings of all of the studies in this treatise have already provided useful information for the design of future experiments in this area. Using the biolistic apparatus, it should be straight-forward to insert any molecule of choice within various transplant combinations (see below).

FUTURE DIRECTIONS

Several points of interest raised by this work are worthy of pursuit. The transfection issues are as follows. Non-viral transfection methods for use in islets are worth developing, both for the immunological and time-frame reasons discussed above. If the biolistic method is to continue to be used, the blast to blast variability has to be improved. This probably means a change in technology from those relying on disc rupture. In fact, Bio-Rad seems to have already realized the limitations of the disc rupture models and is apparently now focusing on developing a different type of helium-based model. In addition, the problem of having to use more limited amounts of each DNA when studying a combination of molecules has to be overcome.

Alternatively, newer non-viral methods of transfection could be explored. Lipofection reagents continue to be improved. A small degree of islet dissociation prior to the lipofection procedure may improve transfection efficiency. By far the highest efficiencies have been obtained using recombinant adenoviruses. The use of adenovirus-polylysine complexes which utilize the adenovirus receptor look to be promising. New methodologies and modifications are appearing at a rapid rate so the outlook for better transfection efficiencies is fairly optimistic. In addition to transfection efficiency, higher levels of gene expression may be obtained with more efficient and/or more tissue-specific promoter/enhancer elements. In the case of CTLA4-Ig, the levels of protein present could be augmented by preincubation of the islets with CTLA4-Ig protein prior to transplantation. In this situation, most or all of the B7 sites on the islet APCs would be initially blocked in vitro and the islets would only have to produce enough protein for ongoing blockade.

The immunological questions raised by these studies are many. Obviously we have not addressed the specific mechanisms responsible for allograft protection. Has anergy in fact been achieved? Does a true state of tolerance exist? What is the nature of the lymphocytic peri-infiltrate? Does it play some sort of active role in graft protection (e.g. are there 'suppresser cells' present)? Could evidence of apoptosis be detected earlier on in the case of the Fas ligand protected allografts? These are all areas which deserve further investigation to answer the mechanistic questions raised.

Finally, now that the tools and procedures have been developed and are in place, it should be relatively easy to extend this approach to other combinations of molcules or to transfer it to other transplantation systems. For example, new molecules or combinations could be tested. The most obvious candidates would be molecules designed to interfere with the other co-stimulatory pathways such as the CD2:LFA-3 or CD40:CD40 ligand interaction. Biolistics could provide a immediate screening method for such combinations, rather than having to await the production of each of the recombinant adenoviruses. Alternatively, the effect of CTLA4-Ig, Fas ligand or both could be examined in terms of the autoimmune processes responsible for the initial onset and recurrence of Type I disease. Their effects could also be investigated within the xenograft arena. Further, this system could be employed to examine the effects of other immune regulating molecules such as cytokines on graft rejection. Complement regulators such as Decay Accelerating Factor (DAF) could be produced to examine their effectiveness in blockade of xenograft destruction. The effects of non-immunologic molecules could also be tested. Such molecules would include islet-specific trophic substances or angiogenic factors such as TGF-ß which may improve engraftment of the transplanted tissue. As our molecular understanding of these processes continues to grow, so does the list of potential candidates for testing within this system.

The usefulness of this study could extend into a wide range of disciplines. Within the larger picture, the biolistic approach which has been developed here could be adapted for use with many types of tissues. Similarly, the molecules which have been cloned for use in this study could be utilized to answer a number of immunological questions unrelated to transplantation. It is hoped that both of these tools will prove useful in the future as the era of gene therapy pertaining to many diverse areas of medicine rapidly approaches.

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APPENDIX

DETAILS OF GENE CONSTRUCTION

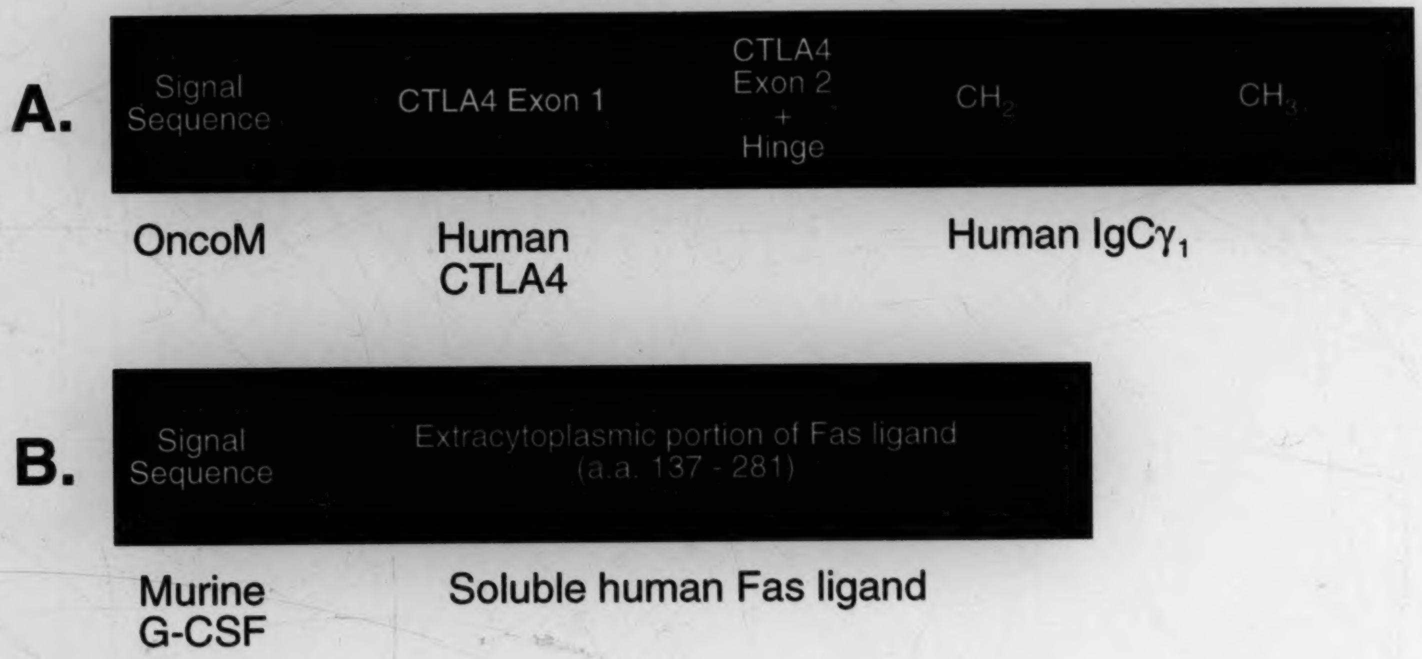
A.
Signal Sequence
CTLA4 Exon 1
CTLA4 Exon 2 + Hinge
CH2
CH3
OncoM
Human CTLA4
Human IgCγ1
B.
Signal Sequence
Extracytoplasmic portion of Fas ligand (a.a. 137 - 281)
Murine G-CSF
Soluble human Fas ligand

Figure A-1. **Schematic of immunomodulatory molecules used in allotransplant studies.**
A. Human CTLA4-Ig showing constitutive sections of the fusion protein gene.
B. Soluble human Fas ligand showing constitutive sections of the truncated molecule.

A series of cassettes were synthesized which were sequentially ligated together. Each cassette contained specific restriction sites that had been introduced by primers which encoded for the appropriate point mutations but which did not affect the amino acid sequence (i.e. non-coding mutations).

1. Exon 1 (excluding the last 3 amino acid residues) of human CTLA4 was cloned from genomic (placental) DNA using PCR with a combination of Taq polymerase and Vent polymerase in a 10:1 ratio. Primers contained specific sites shown below.

   Product 1: Apa I-Xba I-Bst XI-[redacted]-Bgl II-Nar I-Not I

2. The Oncostatin M signal sequence was synthesized by filling in the ends of overlapping oligonucleotides (60mers) which had been previously purified on a 15% acrylamide gel.

   Product 2: Xba I-[redacted]-Bst XI

3. The Onco M signal sequence (Product 2) was ligated into the gene (Product 1) through the Xba I and Bst XI sites.

   Product 3: Apa I-Xba I-[redacted]-Bst XI-[redacted]-Bgl II-Nar I-Not I

4. The proximal portion of Exon 2 (first 8 amino acid residues) spliced to the hinge portion of human IgCγ1 (17 amino acid residues) was synthesized from overlapping oligonucleotides (53mers) as Step 2 above.

   Product 4: Bgl II-[redacted]-Bcl I-[redacted]-Nar I

5. The human IgCγ1 tail was synthesized by PCR from cDNA.

   Product 5: Apa I-Nar I-[redacted]-Not I

6. The hinge cassette (Product 4) cut with Bgl II and Nar I and the IgCγ1 cassette (Product 5) were force cloned by a two step ligation into the gene to form the final product.

Product 6:
Apa I-Xba I-[redacted]-Bst XI-[redacted]-Bgl II-[redacted]-Bcl I-[redacted]-Nar I-[redacted]-Not I

Figure A-2. **Cloning strategy for human CTLA4-Ig**
All enzymes were purchased from New England Biolabs (Beverly, MA).

1. **Exon 1 of human CTLA4.**

Forward: GGGCCCTCTAGAGCCATGCACGTGGCCCAGCCTGC

Reverse: GCGGCCGCGGCGCCAGATCTGGGCTCCGTTGCCTATGCCC

2. **Oncostatin M signal sequence.**

Forward: GCTCTAGAGCCCACCATGGGGGTACTGCTCACACAGAGGACG
CTGCTAAGCTTGGTCCTT

Reverse: ACTCCACGTGCATGGCCATGCTCGCCATGCTTGGAAACAGGAG
TGCAAGGACCAAGCTTA

3. **Exon 2 of human CTLA4 and hinge region of human IgCγ1.**

Forward: AGATCTATGTAATTGATCCAGAACCGTGCCCAGATTCTGATCA
AGAGCCCAAAT

Reverse: GGCGCCGGGGACGGTGGGGATGTGTGAGTTTTGTCAGAAGAT
TTGGGCTCTTGA

4. **Human IgCγ1 tail.**

Forward: GGGCCCGGCGCCTGAACTCCTGGGGGGACCGTC

Reverse: GCGGCCGCTCATTTACCCGGAGACAGGGAGA

Figure A-3. Synthetic oligonucleotides used in synthesis of human CTLA4-Ig.

```
DNA sequence   1175 b.p.   gctctagagccc ... atgagcggccgc   linear

      Xba I  Koziak                                            Hind III
1    gctctagagcccacc ATG GGG GTA CTG CTC ACA CAG AGG ACG CTG CTA AGC TTG GTC CTT GCA     63
1                    M   G   V   L   L   T   Q   R   T   L   L   S   L   V   L   A       16
                                             Bst XI
64   CTC CTG TTT CCA AGC ATG GCG AGC ATG GCC ATG CAC GTG GCC CAG CCT GCT GTG GTA CTG    123
17   L   L   F   P   S   M   A   S   M   A   M   H   V   A   Q   P   A   V   V   L       36

124  GCC AGC AGC CGA GGC ATC GCC AGC TTT GTG TGT GAG TAT GCA TCT CCA GGC AAA GCC ACT    183
37   A   S   S   R   G   I   A   S   F   V   C   E   Y   A   S   P   G   K   A   T       56

184  GAG GTC CGG GTG ACA GTG CTT CGG CAG GCT GAC AGC CAG GTG ACT GAA GTC TGT GCG GCA    243
57   E   V   R   V   T   V   L   R   Q   A   D   S   Q   V   T   E   V   C   A   A       76

244  ACC TAC ATG ATG GGG AAT GAG TTG ACC TTC CTA GAT GAT TCC ATC TGC ACG GGC ACC TCC    303
77   T   Y   M   M   G   N   E   L   T   F   L   D   D   S   I   C   T   G   T   S       96

304  AGT GGA AAT CAA GTG AAC CTC ACT ATC CAA GGA CTG AGG GCC ATG GAC ACG GGA CTC TAC    363
97   S   G   N   Q   V   N   L   T   I   Q   G   L   R   A   M   D   T   G   L   Y      116

364  ATC TGC AAG GTG GAG CTC ATG TAC CCA CCG CCA TAC TAC CTG GGC ATA GGC AAC GGA GCC    423
117  I   C   K   V   E   L   M   Y   P   P   P   Y   Y   L   G   I   G   N   G   A      136
       Bgl II                                            Bcl I
424  CAG ATC TAT GTA ATT GAT CCA GAA CCG TGC CCA GAT TCT GAT CAA GAG CCC AAA TCT TCT    483
137  Q   I   Y   V   I   D   P   E   P   C   P   D   S   D   Q   E   P   K   S   S      156
                                               Nar I
484  GAC AAA ACT CAC ACA TCC CCA CCG TCC CCG GCG CCT GAA CTC CTG GGG GGA CCG TCA GTC    543
157  D   K   T   H   T   S   P   P   S   P   A   P   E   L   L   G   G   P   S   V      176

544  TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA    603
177  F   L   F   P   P   K   P   K   D   T   L   M   I   S   R   T   P   E   V   T      196

604  TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC    663
197  C   V   V   V   D   V   S   H   E   D   P   E   V   K   F   N   W   Y   V   D      216

664  GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC    723
217  G   V   E   V   H   N   A   K   T   K   P   R   E   E   Q   Y   N   S   T   Y      236

724  CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG    783
237  R   V   V   S   V   L   T   V   L   H   Q   D   W   L   N   G   K   E   Y   K      256

784  TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA    843
257  C   K   V   S   N   K   A   L   P   A   P   I   E   K   T   I   S   K   A   K      276

844  GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG    903
277  G   Q   P   R   E   P   Q   V   Y   T   L   P   P   S   R   D   E   L   T   K      296

904  AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG    963
297  N   Q   V   S   L   T   C   L   V   K   G   F   Y   P   S   D   I   A   V   E      316

964  TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC CAC AAG ACC ACG CCT CCC GTG CTG GAC TCC   1023
317  W   E   S   N   G   Q   P   E   N   N   H   K   T   T   P   P   V   L   D   S      336

1024 GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG   1083
337  D   G   S   F   F   L   Y   S   K   L   T   V   D   K   S   R   W   Q   Q   G      356

1084 AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC   1143
357  N   V   F   S   C   S   V   M   H   E   A   L   H   N   H   Y   T   Q   K   S      376
                                       Not I
1144 CTC TCC CTG TCT CCG GGT AAA TGA gcggccgc                                           1175
377  L   S   L   S   P   G   K   *                                                       384
```

Figure A-4. DNA sequence of the human CTLA4-Ig gene construct.

A single non-coding mutation of thymidine for guanosine appears at position 726.

Introduced restriction sites, Koziak sequence and cys to ser substitutions are underlined.

```
DNA sequence    577 b.p.    acgcgtccaccA ... CTAAgcggccgc    linear

  1 acgcgtccacc ATG GCT CAA CTT TCT GCC CAG AGG CGC ATG AAG CTA ATG GCC CTG CAG CTG   62
  1             M   A   Q   L   S   A   Q   R   R   M   K   L   M   A   L   Q   L     17

 63 CTG CTG TGG CAA AGT GCA CTA TGG TCA GGA CGA GAG GCC GTT CCC CTG GTC ACT GTC AGC  122
 18 L   L   W   Q   S   A   L   W   S   G   R   E   A   V   P   L   V   T   V   S     37

123 GCT CTA GAA CCC CCT GAA AAA AAG GAG CTG AGG AAA GTG GCC CAT TTA ACA GGC AAG TCC  182
 38 A   L   E   P   P   E   K   K   E   L   R   K   V   A   H   L   T   G   K   S     57

183 AAC TCA AGG TCC ATG CCT CTG GAA TGG GAA GAC ACC TAT GGA ATT GTC CTG CTT TCT GGA  242
 58 N   S   R   S   M   P   L   E   W   E   D   T   Y   G   I   V   L   L   S   G     77

243 GTG AAG TAT AAG AAG GGT GGC CTT GTG ATC AAT GAA ACT GGG CTG TAC TTT GTA TAT TCC  302
 78 V   K   Y   K   K   G   G   L   V   I   N   E   T   G   L   Y   F   V   Y   S     97

303 AAA GTA TAC TTC CGG GGT CAA TCT TGC AAC AAC CTG CCC CTG AGC CAC AAG GTC TAC ATG  362
 98 K   V   Y   F   R   G   Q   S   C   N   N   L   P   L   S   H   K   V   Y   M    117

363 AGG AAC TCT AAG TAT CCC CAG GAT CTG GTG ATG ATG GAG GGG AAG ATG ATG AGC TAC TGC  422
118 R   N   S   K   Y   P   Q   D   L   V   M   M   E   G   K   M   M   S   Y   C    137

423 ACT ACT GGG CAG ATG TGG GCC CGC AGC AGC TAC CTG GGG GCA GTG TTC AAT CTT ACC AGT  482
138 T   T   G   Q   M   W   A   R   S   S   Y   L   G   A   V   F   N   L   T   S    157

483 GCT GAT CAT TTA TAT GTC AAC GTA TCT GAG CTC TCT CTG GTC AAT TTT GAG GAA TCT CAG  542
158 A   D   H   L   Y   V   N   V   S   E   L   S   L   V   N   F   E   E   S   Q    177

543 ACG TTT TTC GGC TTA TAT AAG CTC TAA gcggccgc                                    577
178 T   F   F   G   L   Y   K   L   *                                                186
```

Figure A-5. DNA sequence of the human soluble Fas ligand gene construct.